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GRANT NUMBER DAMD17-94-J-4241

TITLE: Regulation of Nutrient Transport in Quiescent, Lactating, and Neoplastic Mammary Epithelia

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REPORT DATE: October 1996

TYPE OF REPORT: Annual

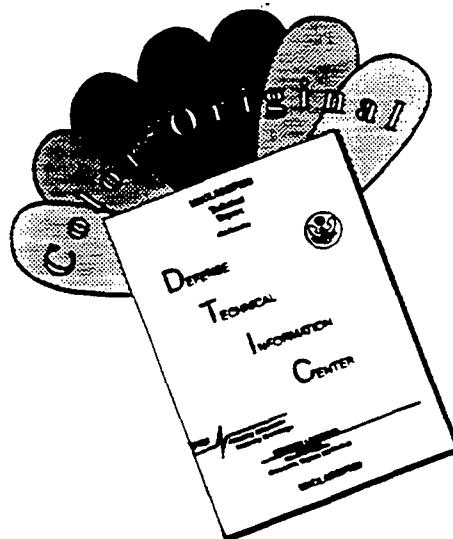
PREPARED FOR: Commander  
U.S. Army Medical Research and Materiel Command  
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1. AGENCY USE ONLY <i>(Leave blank)</i>	2. REPORT DATE October 1996	3. REPORT TYPE AND DATES COVERED Annual (20 Sep 95 - 19 Sep 96)	
4. TITLE AND SUBTITLE    Regulation of Nutrient Transport in Quiescent, Lactating, and Neoplastic Mammary Epithelia		5. FUNDING NUMBERS DAMD17-94-J-4241	
6. AUTHOR(S) Peter M. Haney, M.D., Ph.D.			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Washington University St. Louis, MO 63130-4899		8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick Frederick, Maryland 21702-5012		10. SPONSORING/MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES			
12a. DISTRIBUTION / AVAILABILITY STATEMENT Distribution authorized to U.S. Government agencies only; Proprietary Information, Oct 96. Other requests for this document shall be referred to Comander, U.S. Army Medical Research and Materiel Command, ATTN: MCMR-RMI-S, Fort Detrick, Frederick, MD 21702-5012		12b. DISTRIBUTION CODE	
13. ABSTRACT <i>(Maximum 200)</i>  <b>The specific aims of this proposal are to characterize the role of GLUT1 and other potential glucose transporters in lactating and neoplastic mammary epithelia, to identify novel transporters or sorters, to describe their developmental regulation, and to test possible associations between glucose transport and the neoplastic phenotype. Double-label immunofluorescence and subcellular fractionation by density gradient centrifugation independently demonstrate that GLUT1 is localized in the Golgi in response to the hormonal milieu of lactation, both <u>in vitro</u> and <u>in vivo</u>; corresponding with this, lactose biosynthesis is increased several-fold. Northern and Western blots for GLUT1 and GLUT5 indicate that the developmental regulation of glucose transporters is isoform-specific, and a precipitous decline in GLUT1 levels at weaning appears not to be due to transcriptional effects, but to changes in translational efficiency or GLUT1 protein degradation. Differential display analysis has shown six genes differentially expressed in mammary epithelial cells treated with prolactin and dexamethasone. One of these is lactate dehydrogenase A, and five are novel, and represent potential candidates to explain the Golgi sequestration of GLUT1 observed in secretion medium. The ability to understand and alter the amount of subcellular targeting of GLUT1 may have therapeutic implications in breast cancer.</b>			
14. SUBJECT TERMS    Breast Cancer, Lactation, Glucose, Transport		15. NUMBER OF PAGES 63	
16. PRICE CODE			
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Limited

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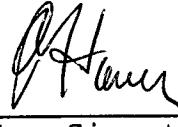
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## Introduction

### Prologue

The Department of Defense review of my October 1995 progress report was accompanied by a request that I rewrite the SOW for the grant and that the 1996 report be reorganized under the new SOW. Note that the specific aims - characterizing the role of GLUT1 and other potential glucose transporters, identifying novel transporters or sorters, describing their developmental regulation, and testing possible associations between glucose transport and the neoplastic phenotype- are unchanged. The rationale for the request for a new SOW was that the following three factors modified my approach to the specific aims of the proposal:

1. The helpful criticism provided in the critiques of my 1993 Career Development Award and New Investigator Award proposals. The most helpful criticisms of the original proposals involved a need to focus on glucose transport and a need to overcome reliance on established cell lines, which may not be fully informative. Therefore, I deferred any experiments on amino acid transport, and planned experiments which rely more on intact gland, acini or organoids, or primary cells in culture.
2. Methodologic advances have led me to give a higher priority to the original Task 4, Identification of novel proteins involved in nutrient transport in lactating mammary epithelia, using the differential display technique, which was not a method originally proposed.
3. Literature reports have affected the priority of pursuing the original Tasks 1 and 2, which involve characterization of GLUT1 and examination of other glucose transporters. Questions about developmental regulation of GLUT1 were substantially answered by Camps et al. (Camps, et al., 1994).

I had also not included the important task of establishing a technique for measurement of lactose biosynthesis in the SOW. Therefore, I submit the following revised Statement of Work. Please note that I consider October, 1995 as month 1.

## Statement of Work

### Regulation of Nutrient Transport in Quiescent, Lactating, and Neoplastic Mammary Epithelia

Task 1, Description of the developmental and hormonal regulation of glucose transport and lactose biosynthesis in CIT3 cells (Months 1-18) and in mammary gland (Months 10-30; about 60 rats)

- a. Characterization of the GLUT1 glucose transporter and rates of lactose biosynthesis in mammary epithelia from four groups (non-pregnant, pregnant, lactating, and involuting) of 15 rats
  - i. confocal immunofluorescent microscopy (3 rats per group)
  - ii. immunogold labelling and electron microscopy (3 rats per group)
  - iii. subcellular fractionation (3 rats per group)
  - iv. determination of apical and basal glucose uptake (6 rats per group)
  - v. lactose biosynthesis
- b. Exclusion of other known glucose transporter isoforms (in same animals)
  - i. Northern blots
  - ii. Western blots

Task 2, Identification of novel proteins involved in glucose transport in lactating mammary epithelia (Months 1-36; about 8 rats)

- a. identification of a novel Golgi glucose transporter (these represent alternative approaches and are listed in the order in which they would be pursued)
  - i. differential display of gene expression in CIT<sub>3</sub> cells in presence and absence of prolactin and dexamethasone and in mammary epithelia isolated from human milk and reduction mammoplasty tissue, to identify candidate genes for unique glucose transporters (or glucose transporter sorters)
  - ii. screening of lactating mammary gland cDNA library by low-stringency hybridization with known isoforms or with oligonucleotides representing highly conserved regions of the five known isoforms
  - iii. PCR-based cloning using oligonucleotides representing highly conserved regions of glucose transporters from a lactating mammary gland cDNA library
  - iv. subtraction cloning to isolate and subsequently characterize differentially expressed transcripts between luminal epithelial cells from human milk and quiescent epithelial cells from reduction mammoplasty
  - v. screening of an expression library from lactating mammary gland with antibodies generated against partially purified Golgi membranes from lactating mammary gland

- b. identification of a novel, hormonally regulated GLUT1 sorter
  - i. see 2.a.i; differential display
  - ii. identification of hormonally regulated GLUT1 sorting motif by analysis of subcellular distribution of chimeric glucose transporters
  - iii. screening of lactating mammary gland expression library with peptide composed of GLUT1 sorting motif, or, use of two-hybrid system using the GLUT1 sorting motif as "bait" (2 rats for each of up to 4 libraries)
- c. demonstration of tissue specificity of Golgi glucose transport to mammary gland by expression of alpha-lactalbumin in HepG2 cells

Task 3. Examination of a possible association between abnormal glucose transport and the neoplastic phenotype (Months 19-36; about 24 mice)

- a. use of techniques listed above to:
  - i. observe differences between non-neoplastic and neoplastic established mammary epithelial cell lines with respect to activity, amount, and subcellular distribution of relevant transporters and other proteins, and their regulatory mechanisms, as identified above
  - ii. determine whether differences observed in Task 3.a.i are also seen in cells derived from human breast tumors
- b. use of non-neoplastic and neoplastic mammary epithelial cells overexpressing heterologous glucose transporters, in the pattern(s) characterized in Tasks 3.a.i and 3.a.2, or overexpressing factors which alter glucose transporter function or subcellular distribution, to examine effects on phenotype, including transport properties, nutrient utilization, growth characteristics, and cellular morphology
- c. use of mammary epithelial cells transplanted into a cleared mammary fat pad after genetic manipulation *in vitro* to confirm findings of Task 3.b and to further characterize changes in phenotype related to cellular organization and growth (up to 8 groups of 3 mice)

### Subject and purpose of the research

Glucose is critical to mammary epithelial cells not only because it serves as a fuel and as a building block for glycoproteins and glycolipids, but also because of its role as the major substrate for the synthesis of lactose and lipid, which together contribute 80-90% of the calories in human milk. Lactose is the major carbohydrate constituent of human milk and the major determinant of its osmolarity, and therefore, of milk production(Neville, et al., 1983). Synthesis of lactose is carried out exclusively within the Golgi apparatus of mammary epithelial cells, in a reaction catalyzed by galactosyltransferase complexed to the tissue-specific protein alpha-lactalbumin(Strous, 1986). Lipid components of human milk, which are also primarily derived from glucose, provide about one-half of the caloric content of milk. Thus, regulation of glucose uptake in mammary epithelia must account for two very different states, the quiescent state, with a relatively small demand for glucose, and the lactating state, with an extraordinary demand for glucose to fuel the high metabolic rate of the epithelial cells themselves as well as to provide substrate for the synthesis of milk to sustain the young.

Breast cancer cells also exhibit an increased demand for glucose, as reviewed below. The molecular mechanisms by which the enhanced transport of this vital nutrient into tumor cells is accomplished require further investigation. Elucidation of the molecular mechanisms by which the mammary epithelia achieves the adaptations in glucose transport needed for lactation, and the examination of their possible dysregulation in neoplastic mammary epithelium, form the central goals of this proposal.

There are two mechanisms for glucose transport into cells. For most cells, the sole mechanism is the passive diffusion of glucose into cells, facilitated by the five isoforms of the glucose transporter family(Burant, et al., 1992, Mueckler, 1994). These are designated GLUT1-GLUT4, and GLUT7, in the order in which they were cloned. (GLUT5 is actually a fructose transporter(Burant, et al., 1992), and GLUT6 is a pseudogene related to GLUT5(Kayano, et al., 1990)). These isoforms exhibit distinct regulatory properties, tissue distributions, and kinetics. However, they are all integral membrane proteins containing approximately 500 amino acids. Hydropathy plots based on amino acid sequences predicted from cDNA sequence suggest that all share a common topology, which includes cytoplasmic N- and C-termini, twelve membrane spanning domains, one exofacial loop which contains an N-linked glycosylation site, and one approximately 65 amino acid intracellular loop midway through the transporter. There is a striking degree of homology among these isoforms, which are 50-65% identical in their amino acid sequence. GLUT1 is also known as the endogenous glucose transporter because of its nearly ubiquitous tissue distribution. It is important in basal glucose uptake and is usually found primarily in the plasma membrane. It is the only glucose transporter isoform convincingly shown to be expressed in mammary epithelia. In the specialized setting of

intestinal(Hwang, et al., 1991) and renal(Miller, et al., 1992, Pajor, 1994) epithelia, and possibly in pulmonary epithelia(Kemp and Boyd, 1992), glucose is also taken up by active transport across specialized membrane domains by tissue-specific isoforms of the sodium, glucose co-transporter(Hediger, et al., 1987). A possible role of this protein in mammary gland glucose transport during lactation has recently been suggested(Shennan and Beechey, 1995).

Mammary gland is unique in its requirement for free glucose within the Golgi, the site of lactose synthesis from glucose and UDP-galactose. The substrates for glycosylation of proteins within the Golgi, which occurs in many cell types, are nucleotide sugars, not free sugars. Wilde and Kuhn measured glucose uptake into rat mammary acini at different glucose concentrations, and directly measured intracellular glucose concentration, to conclude that glucose transport is rate-limiting for lactose synthesis(Wilde and Kuhn, 1981). Madon et al. measured cytochalsin B binding of fractionated rat mammary gland and found that the GLUT1 glucose transporter accounted for only about one-half of the cytochalasin B binding sites of Golgi, strongly suggesting that a unique transporter resides in the Golgi of lactating mammary gland(Madon, et al., 1990). This proposal aims to extend our understanding of glucose transport within lactating mammary gland by identifying and characterizing the molecular species responsible for glucose transport within the mammary epithelial cell and by exploring their developmental and hormonal regulation. This forms a prerequisite for understanding glucose transport in breast cancer.

Seven decades ago, Warburg(Warburg, 1923) appreciated that tumor cells show high rates of glucose uptake, glucose metabolism, and respiration(Hatanaka, 1974, Merrall, et al., 1993). Several lines of evidence suggest the value of a comprehensive understanding of glucose transport in mammary gland in the context of breast cancer. Brown et al.(Brown and Wahl, 1993) showed that higher expression of the glucose transporter GLUT1 by breast cancer cells compared with the healthy breast tissue is common. Several groups have recently shown that the glucose analog 18-F-fluoro-2-deoxyglucose can be used to detect and stage breast cancer(Wahl, et al., 1991, Tse, et al., 1992, Nieweg, et al., 1993, Adler, et al., 1993, Crowe, et al., 1994), suggesting that an abnormally high uptake of glucose is a consistent finding in breast cancer. The MCF-7 line of breast cancer cells was established over twenty years ago(Soule, et al., 1973) and has been characterized in literally hundreds of studies since then. Inhibition of glycolysis in MCF-7 breast cancer cells by extracellular AMP markedly inhibited cell proliferation(Hugo, et al., 1992). Elegant NMR studies have recently shown that, in MCF-7 human breast cancer cells, tamoxifen inhibits glucose consumption and lactate production by 50%, compared to estrogen-treated cells, and that estrogen rescue of tamoxifen treated cells was associated with a rapid increase in glucose consumption(Furman, et al., 1992, Neeman and Degani, 1989). Glucose-6-phosphate dehydrogenase, a key enzyme of glucose metabolism, is strikingly elevated in mammary epithelial cells from patients with breast cancer compared to those with benign breast disease(Barron, et al., 1991). Its activity was also significantly increased in morphologically normal

tissue from cancer-containing breasts when compared to breasts with no known cancer, suggesting the possibility that metabolic abnormalities precede morphological changes in breast carcinogenesis(McDermott, et al., 1990). GLUT1 is the major glucose transporter isoform expressed in mammary epithelial cells. Importantly, GLUT1 is also the only known glucose transporter isoform whose gene is activated at the level of transcription in cells transformed by oncogenes such as *fps*, *src*, and *ras* (Birnbaum, et al., 1987, Flier, et al., 1987). Since this response is direct, the GLUT1 gene is an immediate early gene. Of special interest with respect to breast cancer is that the *neu* oncogene induces synthesis of GLUT1 mRNA and increases glucose uptake three-fold in fibroblasts(Sistonen, et al., 1989). Several groups have reported that between 18.8% and 67% of patients with breast cancer have elevated serum *neu* protein levels or amplification of HER-2/*neu*.(Kath, et al., 1993, Charpin, et al., 1993, Descotes, et al., 1993, Bacus, et al., 1992). Induction of GLUT1 mRNA synthesis also occurs in cells after addition of serum, peptide growth factors, and agents which increase intracellular cAMP concentration(Hiraki, et al., 1989). The two enhancer elements responsible for the responsiveness of the GLUT1 gene to growth factors and oncogenes have been characterized(Muramiki, et al., 1992).

What might the biochemistry and molecular cell biology of glucose transport in lactating mammary gland teach us about breast cancer? One fundamental answer is that, to understand the abnormal state of a cell, one must first understand its normal function and development. A more concrete rationale is suggested by the observation that high rates of glucose uptake and high levels of GLUT1 characterize breast tumors, as noted above. Furthermore, certain proteins important in lactation are also expressed in neoplastic breast tissue but not in normal, quiescent, breast tissue. Serum human alpha-lactalbumin, the mammary-specific protein cofactor that combines with galactosyltransferase to form the complex lactose synthetase, has been proposed as a marker for breast cancer(Thean and Toh, 1990). Similarly, a milk fat globule protein is highly expressed in human breast tumors(Hilkens, et al., 1986, Larocca, et al., 1991). There is also a higher molecular weight glycoprotein detectable in milk and breast carcinomas(Sekine, et al., 1985). None of these are expressed in non-lactating, non-neoplastic mammary gland.

In contrast to the transfer of lactose into milk across apical membrane, which occurs by vesicle fusion and is not carrier-mediated(Neville, et al., 1983), glucose must be transported across at least three distinct cellular membranes of lactating mammary epithelial cells. Specifically, glucose must be transported from the blood across the basal plasma membrane to the mammary gland cytoplasm, from the cytoplasm across the Golgi membrane to the Golgi, where lactose synthesis occurs(Kuhn and White, 1975), and from the cytoplasm across the apical membrane to milk. No known isoforms of the glucose transporter family are known to reside primarily in the Golgi. The regulation of glucose transport must take into account the difference in requirements of the quiescent and the lactating gland. Therefore, the specific hypotheses to be tested are:

## 1. Glucose transport into mammary epithelial cells involves novel proteins.

It seems unlikely that GLUT1 alone can account for the complex nature of glucose transport within lactating mammary gland. GLUT1 is the only known transporter isoform expressed in mammary gland(Madon, et al., 1990), where it is reportedly found in Golgi as well as in plasma membrane. However, the sole method used by Madon et al., subcellular fractionation, results in fractions enriched in plasma membrane and Golgi but nevertheless subject to contamination with other cellular compartments(Kinne-Saffran and Kinne, 1989), and GLUT1 is found in Golgi in no other cell type(Haney and Mueckler, 1994). Isolated Golgi of mammary gland demonstrate high uptake of glucose(White, et al., 1980), and fusion of Golgi membrane with liposomes confers glucose uptake(Wallace and Kuhn, 1986), suggesting the presence of a functional glucose transporter. In other polarized epithelial cells, GLUT1 is targeted primarily to the basolateral membrane, not to apical membrane or to intracellular compartments such as Golgi(Harris, et al., 1992). Nevertheless, I have provided evidence that supports the localization of GLUT1 in Golgi as well as in plasma membrane in cultured cells of an established mouse mammary epithelial cell line treated with insulin, EGF, prolactin, and dexamethasone. This suggests the existence of one or more cell-specific factors conferring the unique subcellular targeting of GLUT1 to Golgi as well as plasma membrane in lactating mammary epithelium. Their identification and characterization would be novel and important. Their mechanism of action might be similar to that recently proposed for the rubella virus E2 glycoproteins, in which a heterodimer of two integral membrane proteins can be retained in the Golgi by a single retention signal(Hobman, et al., 1995). Another possibility is that the cellular sorting machinery is specifically altered to redistribute GLUT1 from the plasma membrane to the Golgi, analogous to the ligand-induced redistribution of a human KDEL receptor from the Golgi complex to the endoplasmic reticulum(Lewis and Pelham, 1992).

Others have provided some evidence that GLUT2 and GLUT4 are not expressed in epithelial cells of lactating mammary gland(Madon, et al., 1990). The presence of GLUT3 and GLUT7 still needs to be excluded. The possibility that the sodium, glucose co-transporter is expressed during lactation, as suggested but not proven by Shennan et al.(Shennan and Beechey, 1995), will be examined. Finally, it is important to determine whether an as-yet unknown glucose transporter plays a role. The hypothesis will be tested by examining whether regulated changes in the activity and the subcellular distribution of GLUT1 in response to the demands of lactation, and under other conditions such as fasting intact animals and manipulation of glucose concentration in cultured cells, can account for the observed transport of glucose across basolateral, Golgi, and apical membranes. The attempt to identify and characterize novel proteins as discussed below also represents a direct test of this hypothesis.

The results of this line of experiments may include the identification of a unique glucose transporter specifically suited for transport of glucose into Golgi, and

the identification of a novel trafficking pattern for GLUT1 that includes at least one tissue-specific, hormonally regulated protein involved in the sequestration of GLUT1 within the Golgi of lactating mammary gland. This may provide the knowledge needed to perturb glucose transport in breast cancer cells.

2. Glucose transport into mammary epithelial cells is subject to a high degree of regulation.

GLUT1 and any other transporter isoforms or other novel proteins identified by pursuing the first specific aim are likely to exhibit developmental and hormonal regulation. The activities of key enzymes of lactation, such as acetyl-CoA-carboxylase, fatty acid synthetase, galactosyltransferase, phosphofructokinase, and isocitrate dehydrogenase, among others, expressed per mg DNA, rise several-fold as lactation commences(Wilde, et al., 1986). It is reasonable to expect coordinate regulation of glucose transporters. Given the crucial importance of successful lactation to continued survival of the species, and given the limited evidence that already exists, I expect to confirm that glucose transport into lactating mammary gland is, in fact, exquisitely regulated, and hope to establish the molecular mechanisms of that regulation.

3. Abnormal glucose transport in mammary epithelial cells (i) is associated with abnormal cell growth, and (ii) may facilitate abnormal cell growth.

Only by comprehensively characterizing glucose transport in normal quiescent and lactating mammary gland will we obtain the data needed to understand the significance of a possible role for altered glucose transport in breast cancer. As noted above, there is substantial evidence linking glucose uptake and metabolism with cancer. I anticipate that the association between glucose metabolism and breast cancer will be confirmed. Using techniques of molecular cell biology, I will be able to test whether specific perturbations of glucose transport, including those which might be found to be associated with cancer, can of themselves confer an abnormal phenotype. I also intend to alter glucose transporter targeting in tumor cells to reduce the amount at the cell surface available for glucose uptake; this will test the hypothesis that abnormally high glucose transport is important to support tumor cell growth. There are currently no data upon which to base a prediction of the results of this experiment, although there is the precedent that the *v-sis* oncoprotein loses transforming activity when targeted to the early Golgi complex(Hart, et al., 1994). Successful reduction of glucose transport into tumor cells might offer new therapeutic possibilities.

Scope of the research

Different experimental models are suitable for different components of this proposal. Some experiments will be carried out in cultured cells, including established mouse mammary epithelial cell lines such as CIT<sub>3</sub> and Comma-1-D, and the neoplastic human mammary epithelial cell line MCF-7, as well as primary

cultures derived from animal or human glands. Established cell lines are well-characterized and useful, but because they reflect only in part gene expression in normal lactating mammary gland and breast tumor(Hall, et al., 1986), experiments limited to established cell lines may not provide realistic answers. Therefore, it is necessary to confirm important conclusions by extensive work in more physiologic models. Human tissue from reduction mammoplasty will be used as the starting material for a normal quiescent mammary epithelial cell cDNA library, while luminal mammary epithelium derived from human milk during the first week of lactation will be used to construct a lactating mammary epithelial cell cDNA library. Contamination with macrophages from milk or myoepithelial cells or fibroblasts from mammary gland will be minimized and cells will be characterized with monoclonal antibodies against keratins(Taylor-Papadimitriou and Stampfer, 1992). Cells derived from these sources will also be used for transient transfection studies. Rats have served as a model for studying the changes associated with mammary development and regression, and I will use them as needed for this purpose, with respect to changes in glucose transport. Adaptations of mammary gland glucose transporters in response to experimental manipulation will also be studied primarily in cultured cells, but key findings will need to be observed in intact rats as well. Secretory acini prepared from rodent mammary gland constitute an important in vitro system for confirming hormonal effects observed in cultured cells. Mice will be used in conjunction with reconstitution of mammary gland using the cleared fat pad technique as described below, providing an elegant method for the study of the consequences of manipulation, using methods of molecular cell biology, of glucose transport on phenotype in vivo. The ability to rapidly and reproducibly reconstitute mammary gland from a population of cells subjected to certain experimental conditions, including genetic manipulation, in vitro, will provide crucial verification of findings from established cell lines and from tumors.

1. Description of the developmental and hormonal regulation of glucose transport in mammary gland.

GLUT1 has been identified in total plasma membrane and Golgi fractions of lactating rat mammary gland. As described above, a single method, subcellular fractionation by differential centrifugation, was used to examine subcellular distribution, and the possibility of contamination with other cellular compartments can not be excluded. I have proposed using several methods to determine the distribution of GLUT1 in lactating rat mammary gland, in primary epithelial cells of this gland in culture(Barcellos-Hoff, 1989), and in CIT<sub>3</sub> and Comma 1-D cells, established mouse mammary epithelial cell lines(Danielson, et al., 1984). Dr. Peggy Neville of the University of Colorado School of Medicine has provided me with CIT<sub>3</sub> cells, which she has selected from Comma-1-D cells for their ability to grow well on filters, form tight junctions, and exhibit polarized transport (personal communication). Rats will be studied at four different stages, non-pregnant-non-lactating, late-pregnant, lactating, and involuting. To accommodate the variety of studies planned, and to be able to detect differences of 20-50% in 2-deoxyglucose uptake, I estimate I will need about 60 rats to perform the proposed studies. Details

of certain procedures used below, namely cell culture, [<sup>3</sup>H]-2-deoxyglucose uptake assay, transfection, Western blot analysis, confocal immunofluorescence microscopy, sucrose density gradient analysis, and immunogold labeling and electron microscopy, are found in my previous publications(Haney, et al., 1991, Haney, et al., 1995). Details for other standard procedures, such as Northern blots, library screening, cDNA and oligonucleotide probe radiolabeling, etc., are found in standard lab manuals(Sambrook, et al., 1989, Berger and Kimmel, 1987).

The subcellular distribution of GLUT1 will be examined in polarized cells. Polarity has been established by growing cells on tissue culture inserts(Parry, et al., 1987). To test the possible role of extracellular matrix in mammary epithelial glucose transport, these inserts can be coated with basement membrane matrices such as those derived from EHS tumors, which have been shown to influence gene expression and differentiated functions(Aggeler, et al., 1991, Lin, et al., 1995). The initial approach to subcellular distribution was using confocal immunofluorescent microscopy, which results in sensitive and specific staining of GLUT1 protein. Cells were exposed to specific affinity-purified antibodies, and then to appropriate fluorescent secondary antibodies. Through careful selection of antibody concentrations to be used, the possibility of non-specific staining was minimized, then ruled out using appropriate controls. In this way, it is possible to localize a specific protein to basolateral membrane, apical membrane, Golgi membrane, or another intracellular compartment. An important limitation of this method is that it does not yield quantitative data. Basolateral membrane and apical membrane will be distinguishable(Sjaastad, et al., 1993) because of the polarity of cells grown on filters and because of the nature of confocal microscopy, which visualizes only one section of a cell at a time. Apical or basal membrane markers will be visualized with a second fluorescent antibody(Ellis, et al., 1992). Golgi can be stained using antibodies specific for the Golgi markers alpha-mannosidases I and II(Velasco, et al., 1993, Antony, et al., 1992). Brefeldin A is a fungal metabolite that causes disassembly of Golgi(Klausner, et al., 1992); failure of GLUT1 to alter its subcellular distribution after treatment with Brefeldin A would suggest it was not a Golgi resident(Berger, et al., 1993). Confocal immunofluorescent microscopy will also be applied to sections from lactating and non-lactating rat mammary gland to determine whether the subcellular localization of GLUT1 changes with differentiation.

Immunogold labeling and electron microscopy have been useful tools in defining the intracellular compartments in which glucose transporters are sequestered and in quantitating their subcellular distribution(Haney, et al., 1995, Slot, et al., 1991, Slot, et al., 1991). The distribution of endogenous GLUT1 under different conditions, such as quiescence, lactation, and neoplasia, and the distribution of heterologous transporters or other proteins of interest in experiments described below, will be studied using this method.

Subcellular distribution has been confirmed by subcellular fractionation using iodixanol density gradient centrifugation. The distribution of GLUT1 across the gradient will be directly compared with the distribution of the plasma membrane

marker, 5'-nucleotidase, and the Golgi marker, alpha-mannosidase I. Protocols exist for separation of apical and basolateral plasma membrane fractions in other polarized epithelial cells(Ellis, et al., 1992, Mircheff, 1989). I intend to adapt these to mammary epithelium as well.

Glucose transport activity is quantitated by measuring uptake of radiolabeled 2-deoxyglucose, a non-metabolizable analog of glucose. The use of 2-deoxyglucose presumes that the activity of hexokinase is not rate-limiting for glucose accumulation. The uptake of radiolabeled 3-O-methyl glucose, which is not affected by hexokinase activity, will also be measured under certain conditions, when it is necessary to prove that hexokinase is not influencing results. The amount of GLUT1 protein will be quantitated by Western blotting. Cells will be grown on filters so they are polarized; uptake will be measured from the apical and basolateral surfaces independently. Uptake into intact Golgi isolated by subcellular fractionation will also be measured(McDermott, et al., 1990). These determinations will be interpreted in light of the subcellular distribution of GLUT1 as determined by confocal immunofluorescent microscopy, immunogold labeling and electron microscopy, and subcellular fractionation. Substantial glucose transport activity of a cellular membrane which contains little or no GLUT1 would suggest that another glucose transporter is playing a role.

The possibility that other glucose transporter isoforms might be found in mammary gland has not been rigorously excluded. The simplest way to do so is to attempt to detect the mRNA for the other known transporters, GLUT2, GLUT3, GLUT4, GLUT5, GLUT7, and the sodium, glucose cotransporter, by Northern blotting rat mammary gland poly(A)<sup>+</sup> RNA with cDNA for each transporter. As described above, rats will be studied at four different stages, non-pregnant-non-lactating, late-pregnant, lactating, and involuting. Similar studies will be performed using neoplastic human breast tissue, and established cell lines representing both non-neoplastic (CIT<sub>3</sub>) and neoplastic (MCF-7) states. Results will be confirmed by performing Western blots. The expression of these isoforms will also be studied in cells isolated from human milk(Lindquist, et al., 1994), and in cells isolated from reduction mammoplasty specimens. Dr. Mike Mueckler of the Washington University School of Medicine has supplied me with cDNAs for all known isoforms. If another isoform is detected, its abundance, subcellular distribution, and activity will be studied as described above for GLUT1. Dr. Mueckler has also supplied me with the available specific antibodies for all isoforms.

Established cell lines as well as cells in primary culture will be exposed to factors known to influence mammary epithelial cells, such as prolactin, growth hormone, and insulin-like growth factors(Peters and Rillema, 1992, Flint, et al., 1992, Barber, et al., 1992), among others, to determine whether there is any direct effect on glucose transport. The amounts of glucose transporters can be increased by DNA-mediated transfection of expression vectors which result in their synthesis at high levels; an increase in lactose synthesis in cells expressing supraphysiologic levels of

a specific glucose transporter would confirm that glucose transport limits lactose synthesis and milk production.

## 2. Identification of novel proteins involved in glucose transport in lactating mammary epithelia.

The preliminary data I have provided is consistent with the localization of GLUT1 to Golgi as well as to plasma membrane in lactating mammary gland. The mechanism of this cell-type specific targeting to Golgi will be fascinating to explore. I hypothesize that a tissue-specific sorter must interact with GLUT1 to accomplish this. This interaction must depend on a unique structural determinant of GLUT1. It is important to note that this structural determinant of GLUT1 would not correspond to constitutively active Golgi targeting signals currently under intense investigation in other labs(Saberan-Djoneidi, et al., 1995, Masibay, et al., 1993, Nothwehr, et al., 1993), but to a hormonally regulated Golgi targeting motif. Identification of that determinant will provide a tool for the identification of the sorter. This will be done by expressing chimeric glucose transporters(Haney, et al., 1995) in cultured mammary epithelial cells. Mammary epithelial cells, specifically, MCF-7 cells, have been transfected to study folate metabolism(Chung, et al., 1993)and drug resistance(Chu, et al., 1990). To transfet L6 myoblasts in my recent studies of structural determinants of GLUT4 targeting, I have used calcium-phosphate mediated DNA transfection to generate stable transfectants using Rc/CMV as the expression vector(Haney, et al., 1995). The identical procedure and the identical expression vector were used for transfection of MCF-7 cells(Chung, et al., 1993). This system will allow me to conveniently overexpress not only GLUT1, the transporter normally present in mammary epithelium, but also GLUT4 and any of the other seventeen potentially informative glucose transporter chimeras I have already made and subcloned into Rc/CMV (see Table 1) composed partly of human GLUT1 and partly of rat GLUT4. The targeting and glucose transport activity of chimeric glucose transporters expressed in stable transfectants of an established mammary cell line, such as CIT<sub>3</sub>, can be characterized using all of the methods I have described above. Any desired chimera between GLUT1 and any other isoform can be prepared using recombinant PCR(Haney, et al., 1995, Vallette, et al., 1989). Constructs are sequenced to verify that no errors occurred during PCR. I have incorporated a species-specific monoclonal antibody epitope in the chimeras, so that they can be distinguished from endogenous transporters. In myoblasts, I have used these and other chimeras to show by confocal immunofluorescent microscopy and immunogold electron microscopy that a necessary but not sufficient structural determinant of GLUT4 targeting lies between amino acids 479 and 490, located in the cytoplasmic C-terminal tail (Haney, et al., 1995). Any chimera which does not contain this signal should be useful in the study of GLUT1 targeting in mammary epithelial cells. Importantly, another expression vector utilizing the CMV promoter has also been used successfully in transfection of normal human mammary epithelial cells derived from reduction mammoplasty(Tomasetto, et al., 1993),

suggesting a convenient system for demonstration that targeting domains identified in established mouse cell lines are relevant to human biology.

This hormonally regulated Golgi targeting motif of GLUT1 will be used to clone genes for other presumably cell-type specific proteins involved in the sorting of proteins containing this motif. A radiolabeled peptide encompassing the motif will be used to screen a lactating mammary gland cDNA expression library, and positive colonies will be characterized. Alternatively, the two-hybrid system(Chien, et al., 1991), which identifies proteins interacting with a protein of interest by functional restoration of a eukaryotic transcriptional activator in yeast, and which results in the immediate availability of the cloned gene, could be used. The motif identified using the chimeric glucose transporters described above would be used as the "bait" in the two-hybrid system. In similar applications, this technique has recently been used to identify proteins interacting with the C-terminal peroxisomal targeting signal (PTS1)(Fransen, et al., 1995) and to screen for proteins interacting with the kinase-like domain of the atrial natriuretic peptide (ANP) receptor/guanylyl cyclase(Chinkers, 1994). The regulation of mammary cell-specific proteins that alter glucose transporter targeting to provide substrate for lactose synthesis would provide insights not only into the molecular regulation of milk production but also into mechanisms of the hormonal regulation of cellular sorting machinery.

Madon et al. strongly suggest the possibility of a unique Golgi glucose transporter which binds cytochalasin B(Madon, et al., 1990). Cytochalasin B is a fungal metabolite which binds specifically to the five isoforms of the facilitated diffusion glucose transporter family(Thorens, et al., 1990) but does not bind to the misnamed GLUT5, which is actually a transporter of fructose, not glucose(Burant, et al., 1992). It also does not bind to the sodium- glucose cotransporter, or to other membrane proteins. Therefore, a strategy based on homology with other known isoforms of the facilitated diffusion family, which are 55-75% identical, is an attractive approach to cloning a unique Golgi glucose transporter. This will involve the screening of a cDNA library from lactating mammary gland, either by 1) low-stringency hybridization using known glucose transporter isoforms as probes, 2) low-stringency hybridization with an oligonucleotide representing a region highly conserved across all known isoforms, or 3) PCR using oligonucleotides representing highly conserved regions of known isoforms(Schuchman, et al., 1990). Some of the putative clones obtained by any of these methods would undoubtedly represent GLUT1, but some should also represent a novel glucose transporter, provided homology does indeed occur.

Failure of the above approaches to identify suspected novel transporters, or failure of cytochalasin B to inhibit a glucose transport activity, would suggest that the suspected glucose transporter is not homologous to known transporters. In this case, partially purified Golgi membranes enriched in glucose transport activity prepared from lactating mammary gland would be used to generate antibodies in rabbits. These antibodies would be used to screen an expression library from

lactating mammary gland. This strategy was used successfully to clone GLUT7, a hepatic microsomal glucose transporter(Waddell, et al., 1992). Positive clones would represent Golgi membrane proteins; in lactating mammary gland, this population should include proteins responsible for glucose transport into Golgi. Clones would be tested for their ability to confer glucose uptake by Golgi isolated from transfected mammary epithelium. Any novel transporter would be characterized by determining its apparent molecular weight by SDS-PAGE and the size of its mRNA by Northern blotting. Its amino acid sequence would be predicted based on the nucleotide sequence of the putative clone and used to predict membrane topology based on a hydropathy plot. The sequence would be examined by searching computerized databases for homology with other proteins. The portion of the transporter determining its targeting to Golgi would be identified using chimeric constructs as described above.

Human milk cells can be obtained from milk during the first week of lactation(Taylor-Papadimitriou and Stampfer, 1992), and mRNA can be conveniently isolated(Lindquist, et al., 1994). These are luminal epithelial cells and should contain mRNA for all proteins required for glucose transport, milk component synthesis, and milk secretion. Mammary cells obtained from reduction mammoplasty will contain mRNAs only for proteins involved in "housekeeping" functions. By isolating mRNA from each cell population and constructing a subtracted cDNA library, the likelihood of identifying cDNAs for proteins of interest will be substantially improved(Briehl and Miesfeld, 1991, Feng and Liau, 1993). In the case of technical difficulties, an analogous procedure could be followed in rats, using mammary gland epithelia from lactating and non-pregnant, non-lactating rats; this would require about six rats. The recently described technique of differential display (see below) offers certain advantages over these approaches, and we have pursued it with success. Some of the positive clones may represent novel proteins with unknown functions. In such a case, the cDNA sequence will be examined for homology to known proteins. Hydropathy plots will be constructed, and particular attention will be focused on integral membrane proteins. These will be characterized with respect to developmental and hormonal regulation of the mRNA. Functions suggested by structural homology or regulatory patterns in common with known proteins will be investigated. Promising candidates can be studied further using transfection of their cDNAs in mammary epithelia to observe not only the subcellular localization of the novel protein, but also effects on cellular phenotype, such as altered glucose transport, growth characteristics, or morphology. Expression in *Xenopus* oocytes would be used to define kinetic properties of putative transporters(Mueckler, 1994, Miyamoto, et al., 1995). A possible association of a novel protein with breast cancer will also be investigated by quantitating its levels in established neoplastic mammary epithelial cell lines.

The recently described technique of differential display offers another route to understanding changes in gene expression during lactation, and has already been applied to mammary epithelial cells to identify potential markers for breast cancer(Liang, et al., 1992, Zhang and Medina, 1993, Watson and Fleming, 1994) and

mammary epithelial cell senescence(Swisselm, et al., 1995) and to identify a candidate tumor suppressor gene(Sager, et al., 1993). Preliminary results from our use of this technique in mouse mammary epithelial cells in culture are mentioned below. Since many breast cancer markers are also expressed during lactation, as described above, our use of this technique to identify genes differentially expressed during lactation may identify new markers for breast cancer. I will systematically evaluate this possibility for each identified gene by Northern blotting mRNA isolated from breast cancer specimens. Marlys Schuh, M.D., of the Department of Surgery at Washington University, is collaborating with me in this aspect of the study and will supply up to 20 breast cancer specimens per year as needed.

3. Examination of a possible association between abnormal glucose transport and the neoplastic phenotype.

Glucose transport in MCF-7 breast cancer cells, which exhibit polarized expression of membrane glycoproteins(Zou, et al., 1989), will be characterized by the methods described above. This will include comparisons of hormonal responsiveness of glucose transport. This observational study will describe differences between two established cell lines, the CIT<sub>3</sub> and MCF-7 lines, but differential expression of transporters or other regulators between the two lines, while suggestive, cannot prove the importance of a given protein.

Brown et al.(Brown and Wahl, 1993) examined twelve breast tumors and showed by immunohistochemistry that higher expression of the glucose transporter GLUT1 by breast cancer cells compared with the healthy breast tissue is common. I intend to carry out these studies quantitatively, at the level of mRNA as well as protein, in order to understand the magnitude of the changes in glucose transport seen in neoplasia. Marlys Schuh, M.D., of the Department of Surgery at Washington University, is also collaborating with me in this aspect of the study; the up to 20 breast cancer specimens per year referred to above will also be studied to determine patterns of glucose transporter expression in breast cancer. I anticipate studying at least 20 specimens before drawing conclusions.

The observational approach outlined in the previous paragraph can not distinguish whether changes in glucose transporter expression, which are, after all, likely to be observed, are central and necessary phenomena, or simply epiphenomena. The relationship between expression of a specific glucose transporter and the neoplastic phenotype will therefore be directly tested by stable transfection as described above. Normal mammary epithelial cells will be stably transfected with expression vectors containing the non-inducible CMV promoter, and will express the heterologous transporter constitutively. This will directly test the link between the transporter in question and changes in phenotype, including altered transport properties, glucose utilization, synthesis of milk components, growth characteristics, and cellular morphology. Overexpression in neoplastic cells of factors which might alter glucose transporter activity, such as the factor responsible for intracellular sequestration of GLUT1 in lactating mammary gland,

will directly test the importance of elevated glucose transport activity in contributing to tumor growth. Glucose transporter levels can also be reduced up to 80% using antisense RNA methods(Valera, et al., 1994), providing another avenue for determining the significance of GLUT1 overexpression for the neoplastic mammary cell phenotype. An expression vector based on the mouse mammary tumor virus promoter, which is active in mammary gland(Gunsburg and Salmons, 1986), confers highly inducible synthesis of heterologous proteins in epithelial cells(Hirt, et al., 1992). This will be useful in ruling out any effects of constitutive expression on membrane trafficking or differentiation, and in establishing more firmly the link between expression of heterologous protein and changes in phenotype.

An elegant method for reconstitution of mouse mammary gland from mammary epithelial cells(DeOme, et al., 1959, Medina, 1973) has been adapted to cells genetically altered in vitro(Edwards, et al., 1992) in order to study physiological and morphological correlates of oncogene expression. Normal mammary epithelial cells are isolated from one mouse and briefly put into primary culture, where a gene is introduced by retroviral infection; the cells are then transplanted into the mammary fat pad of a mouse from which the endogenous epithelium has been removed. The transplanted cells grow to reconstitute a "tree" of glandular epithelium. Transplants carrying the Wnt-1 oncogene grew in a hyperplastic pattern, showing abundant fine side-branches, but without development of alveoli. The same authors also showed that expression of the neu/erbB-2 oncogene induced epithelial abnormalities similar to human atypical hyperplasia and sclerosing adenosis(Bradbury, et al., 1993). This will be a useful method to study specifically in vivo the consequences on cellular organization and function of the overexpression of transporters or other unique factors identified in the course of this study. These studies should require about 24 mice, depending on the number of genetic manipulations to be studied. I will also explore the possible use of this system to understand the impact of these genetic alterations on lactose synthesis in vivo. The results of experiments with reconstituted mammary glands will serve to validate, or to question the significance of, findings from established cell lines or tumors.

Background of previous work (September 1994-September 1995)Differential display

Identification of novel proteins involved in nutrient transport in lactating mammary epithelia is one of the central goals of this proposal. I began to pursue this using the differential display technique, which was not a method originally proposed. This method is essentially a systematic comparison of all gene expression in a given cell type under two distinct conditions. It became clear to me since submitting the original proposal that this method involves several advantages over other methods that were included in the proposal. These include power and reproducibility, as well as ease of manipulation of partial cDNA clones of candidate genes. Others have applied this technique to study differences in gene expression between breast cancer cells and non-malignant mammary epithelial cells(Liang, et al., 1992, Zhang and Medina, 1993, Watson and Fleming, 1994), to identify alpha-6 integrin as a candidate tumor suppressor gene(Sager, et al., 1993), and to demonstrate enhanced expression of an insulin growth factor-like binding protein (mac25) in senescent human mammary epithelial cells and its induction with retinoic acid(Swissheilm, et al., 1995). During the first year of this grant, we succeeded in using this complex but powerful technique to identify genes differentially expressed in CIT<sub>3</sub> mammary epithelial cells in response to prolactin and hydrocortisone. Cells were grown to confluence and maintained for five days in either growth medium, which contains insulin and EGF, or in secretion medium, which in addition to these, contains hydrocortisone and prolactin as well, simulating the hormonal milieu of lactation. mRNA was isolated and used as described by the above investigators for differential display, which involves RT-PCR using 24 upstream and 9 downstream primers using RNA from cells grown under two different conditions, for a total of 432 RT-PCR reactions in the initial analysis. Promising cDNAs were chosen for further study to confirm their differential expression. At the time I submitted last year's report, I stated that we were planning to further study nine cDNAs which were significantly induced, and eight cDNAs which show much lower abundance, in secretion medium relative to growth medium.

CIT<sub>3</sub> cells - polarity and differentiation of function

Non-polarized CIT<sub>3</sub> cells were grown on tissue culture plastic, and on Matrigel coated tissue culture plastic, and polarized CIT<sub>3</sub> cells were grown on uncoated tissue culture inserts. Cells grown in Matrigel, a basement membrane matrix shown to provide a more physiological environment and more differentiated function to cultured mammary epithelial cells, and cells grown on inserts, therefore polarized and functioning in a more physiological manner, expressed less GLUT1. Also, secretion medium increased GLUT1 in cells grown on plastic but not necessarily in cells grown under more physiological conditions. 2-Deoxyglucose uptakes of polarized CIT<sub>3</sub> cells on

uncoated tissue culture inserts, grown in growth medium and in secretion medium, demonstrated that glucose transport activity is highly polarized in secretion medium, but not in growth medium. I concluded that mammary epithelial glucose transport is exquisitely sensitive to extracellular milieu, including the basement membrane matrix and the degree of cell polarity permitted.

## Body

### Experimental methods

Immunofluorescent microscopy- Cells were grown in growth medium, which contains insulin at 10 mcg/ml and EGF 5 ng/ml, and in secretion medium, which also contains prolactin 3 mcg/ml and hydrocortisone 3 mcg/ml. Cells were grown on cover slips, treated with BODIPY-TR and DAPI, rinsed, and treated with glycine, Triton X-100, and 2% horse serum. Cells were then exposed to 10 mcg/ml F350, a highly specific and well characterized antibody to GLUT1, overnight. After rinsing, cells were treated for one hour with FITC-labelled goat anti-rabbit IgG F(ab)2, and rinsed. Coverslips were mounted on slides and viewed on an Olympus iX70 microscope equipped with filter cubes appropriate for DAPI, FITC, and Texas Red. Images were acquired with each cube using a black and white Cohu CCD camera, and false color was applied and images superimposed in Adobe Photoshop.

Iodixanol density gradient centrifugation- Cells and mammary glands were homogenized with five strokes of a Dounce homogenizer. Centrifugation at 3000 g, 17000 g, and at 100000 g was used to prepare fractions. The 17000 g pellet, also known as the light mitochondrial pellet, was subjected to a 10-37% iodixanol density gradient. The upper two to three fractions of the gradient were collected and analyzed, along with prior fractions, by SDS-PAGE and Western blotting with F350 to specifically detect GLUT1.

Lactose assays- Lactose in tissue culture medium was assayed using beta-galactosidase and beta-galactose dehydrogenase and measuring the increase in NADH using a kit purchased from Boehringer Mannheim.

Lactose biosynthesis- Cells were incubated with 6-3H-glucose, 3 mcCi/ml, for 1 hr prior to iodixanol density gradient centrifugation. Aliquots of whole homogenate and of Golgi fractions were subjected to liquid scintillation counting and protein assay using a modified Lowry method.

Differential display- Total RNA populations were isolated from cells grown in growth medium and secretion medium, treated with DNase, and used to generate cDNA using anchored primers and 35-S dATP. PCR was carried out using the same anchored primers, and products were subjected to 6% non-denaturing acrylamide gel electrophoresis. Upregulated bands were reamplified, the band was cut out of an agarose gel and subcloned into TA vector or into pAMP10, and sequenced using Sequenase (Amersham).

RNA probe synthesis- The clone containing the insert of interest was linearized by restriction enzyme treatment of the TA or pAMP10 vector

containing it and isolated by agarose gel electrophoresis. Using T7 or SP6 polymerase and alpha-32-P dCTP, an in vitro RNA transcript was prepared for use as a probe.

**Northern and Southern blots-** Northern blots were performed on 5-20 mcg/lane of RNA in formaldehyde agarose gels , blotted onto nylon transfer membranes using SSC, and probed with 32-P RNA or DNA probe (Maniatis, 1989). cDNAs were generated from RNA, subjected to agarose gel electrophoresis, and probed with 32-P cDNA or DNA (Maniatis, 1989).

### Results and discussion

The revised Statement of Work included with this report indicated that Tasks 1 and 2 were to be the focus of research during this grant period. Task 1 corresponds to studies of the developmental regulation of glucose transporter targeting discussed below. Under Task 1, data is provided relative to Subtasks a.i, a.iii, and a.v, and b.i and b.ii. Task 2 corresponds to the use of differential display to identify novel proteins potentially involved in glucose transport, as discussed below. Under Task 2, data is provided relative to a.i. and b.i.

### Developmental regulation of glucose transporter targeting

I have previously shown, in grant submissions and last year's progress report, that CIT3 cells, a mouse mammary epithelial cell line, demonstrate hormonal regulation of the amount, activity, and subcellular targeting of glucose transporters. Prolactin and dexamethasone cause an increase in GLUT1, but not an increase in plasma membrane glucose transport activity. Immunogold electron microscopy and single-label confocal immunofluorescence showed that this was due to an increase in intracellular targeting of GLUT1, but these studies did not reveal a specific intracellular localization. The crucial question is whether the intracellular compartment to which GLUT1 is targeted is the Golgi, since the Golgi apparatus is the site of lactose synthesis from free glucose.

Figure 1 demonstrates that GLUT1 and the Golgi marker BODIPY-TR colocalize in cells grown in secretion medium but not in growth medium (Please note that this is a color figure, and it will not be possible to interpret a black and white copy). In this figure, yellow in the lower panels indicates colocalization, and is seen in secretion medium, while distinct red and green signals predominate in growth medium.

This conclusion is reinforced by Figure 2, which demonstrates subcellular fractionation by density gradient centrifugation of CIT3 cells grown in growth medium (upper panel) and in secretion medium (lower

panel). The results confirm that secretion medium causes an increase in the amount of GLUT1, as previously shown, but also demonstrate localization of GLUT1 in Fractions 1-3, corresponding to Golgi, in significant quantities only in secretion medium. This serves to confirm, by an entirely independent experimental method, the conclusion drawn from the immunofluorescence studies that the intracellular compartment to which GLUT1 is targeted is indeed the Golgi.

That these findings are relevant to lactating mammary gland is demonstrated in Figure 3, which represents subcellular fractionation by density gradient centrifugation of mammary glands of lactating mice 17 days after giving birth. The upper panel shows an actively lactating mouse, while the lower panel represents a mother whose pups were withdrawn 24 hours previously. Note the abundant expression of GLUT1 in the lactating mouse, and the very low levels of GLUT1 present in the weaned mouse. This is an interesting finding, and suggests both that GLUT1 synthesis is acutely regulated and that its degradation is rapid. Of central importance to the question being asked, note that the signal from Golgi (fractions 1 and 2) is very strong in the lactating mouse, but that GLUT1 is virtually undetectable in Golgi from the weaned mouse. This is consistent with the results from CIT3 cells, suggesting that CIT3 cells do represent a good model system. Taken together, these studies in CIT3 cells and in lactating mammary gland indicate that GLUT1 targeting is regulated in order to support the metabolic demands of lactation.

I have also begun examining the developmental regulation of glucose transporters in mouse mammary gland at the mRNA level. Figure 4 demonstrates Northern blots for GLUT1, indicating that GLUT1 message is abundant in both day 10 and 17 lactating mice. In contrast, little GLUT1 mRNA is seen in virgin gland. Interestingly, mice weaned on day 10, as described in the previous paragraph, showed similar levels of GLUT1 mRNA to lactating mice, although weaning causes a precipitous drop in the level of GLUT1 protein. This finding does not support an effect of weaning on transcription of the GLUT1 gene, and suggests rather an effect on the efficiency of translation of the GLUT1 mRNA or a very significant reduction in the half-life of the GLUT1 protein, or both. Figure 5 shows that GLUT5 mRNA is present at very low levels and does not exhibit the developmental regulation seen for GLUT1. Interestingly, significant levels of GLUT5 mRNA are seen in CIT3 cells in both growth and secretion medium. Data regarding levels of GLUT5 protein are not yet available. The GLUT1 studies make clear that one should not assume that glucose transporter protein levels reflect mRNA levels in mammary gland.

Measurements of lactose levels in tissue culture medium, both from CIT3 cells, and from primary mammary epithelial cells derived from mouse mammary gland and from human milk, have been consistently

disappointing. As shown in Table 1, I have never detected a significant concentration of lactose in media from any of these cells, even when cells are grown in the presence of Matrigel, which mimics basement membrane and stimulates mesenchymal-epithelial interactions, and even when cells are grown on inserts and polarize. Since demonstration that glucose transporters are rate-limiting for lactose synthesis requires that lactose biosynthesis be measurable and that effects of altering glucose transport can be proven, this is a serious shortcoming. I am approaching this by continuing to seek conditions that result in measurable lactose levels, but also by employing a more sensitive technique which actually measures lactose biosynthesis from radiolabelled glucose (see Experimental Methods). Table 2 demonstrates that, at least on crude homogenates, this method is capable of detecting a five-fold increase in lactose biosynthesis in Golgi of CIT3 cells grown in secretion medium compared to growth medium. Since these data are based on whole homogenates and fractions, and not on TLC-purified lactose, they must be regarded as representing upper limits of lactose synthesis. This is promising, and I am pursuing lactose purification by TLC so that I can use this method quantitatively.

Identification of genes differentially expressed in response to prolactin and dexamethasone

In last year's report, I outlined that our differential display analysis of CIT3 cells grown in secretion medium compared to growth medium had yielded nine potential up-regulated genes, and eight potential down-regulated genes, that would be candidates for further analysis. In order to identify genes that might play a role in altering glucose transporter targeting in cells grown in secretion medium, we have focused on the up-regulated genes. I acknowledge that down-regulated genes might also be important, in the sense that a candidate gene might actually prevent GLUT1 retention in Golgi, and its absence in secretion medium might therefore promote GLUT1 retention in Golgi. However, based on GLUT1 behavior in all other cell types, I consider this a less likely scenario.

To avoid expending effort on candidates that were not reproducibly up-regulated, we repeated the differential display analysis a total of three times before proceeding to isolation of individual clones. Of the original nine "up" candidates, six met this criterion and were pursued. Each of these has been isolated, subcloned, and sequenced, and GenBank searches have been performed. Of the six, up-regulation has been confirmed using RNA probes made from in vitro transcription of PCR products in two cases, and by Southern blot using cDNAs from CIT3 cells in growth medium and in secretion medium, in three cases. We have not yet had the opportunity to confirm the remaining clone using either of these methods. Up-regulation of the six candidates is shown in Figure 6. Of the six candidates, five represent novel genes, and one appears to be the A (muscle) subunit of lactate

dehydrogenase. Genbank search highlights for each are included in the appendix, and are briefly discussed in each of the following paragraphs.

D6(5)H, consisting of 32 nucleotides, is upregulated in secretion medium compared to growth medium and is abundant in lactating mammary gland of rat, as demonstrated by hybridization with an RNA probe generated by in vitro transcription of the PCR product (Figure 6a). The Genbank BLASTN search shows that this nucleotide sequence has no significant relationship to any known sequence. Interestingly, the BLASTX search revealed, by detecting a resemblance to the class II histocompatibility antigen HLA-DR beta chain, that this clone may potentially code for a dileucine, and I recognize a strong resemblance, also shown in the appendix, between this potential amino acid sequence and the GLUT4 C-terminus, which contains the unique GLUT4 targeting signal. While intriguing, it is impossible to know the significance of this until a full length clone has been identified and it is established whether this is actually the appropriate reading frame.

D6(5)#8, consisting of 253 nucleotides, is upregulated in both lactating and weaned mouse mammary gland, compared to virgin gland, as demonstrated by hybridization with an RNA probe generated by in vitro transcription of the PCR product (Figure 6b). The Genbank BLASTN search shows that this nucleotide sequence has no significant relationship to any known sequence. The BLASTX search does show several potentially significant similarities at the amino acid level, including to a mitochondrial NADH dehydrogenase. Again, it is impossible to know the significance of this until a full length clone has been identified and it is established whether this is actually the appropriate reading frame.

D6(9), consisting of 36 nucleotides, is reproducibly upregulated in secretion medium compared to growth medium, as shown in the DD-PCR reaction (Figure 6c). The BLASTN search shows that the nucleotide sequence bears a resemblance to guanylate cyclase, but the BLASTX search showed no significant homologies at the amino acid level.

D6(14)A, consisting of 44 nucleotides, is upregulated in secretion medium compared to growth medium, as shown in the Southern blot using cDNA from cells grown in growth medium and in secretion medium (figure 6d). This is the only candidate of the six which corresponds to a known gene. It appears to represent a polymorphism, with only one nucleotide different of its 44, from the LDH-A gene, as shown by the BLASTN search. This suggests that LDH may play an important role in lactation; I am planning to confirm by enzyme assay and protein detection that this observation has physiological significance. No study to date has examined a possible role for LDH in supporting the metabolic demands of lactation. Interestingly, breast discharge

LDH-A levels have been suggested as a possible diagnostic tool for detecting breast cancer(Kawamoto, 1994).

D9(14)#63, consisting of about 500 nucleotides, is upregulated in secretion medium compared to growth medium, as shown in the Southern blot using cDNA from cells grown in growth medium and in secretion medium (Figure 6e). We have 124 nucleotides of sequence from the T7 primer and 62 bases from the SP6 primer. Both of these sequences include stretches of 10-20 A or T and these areas may represent cloning artefacts. Neither searches of the entire sequence nor of portions excluding potential cloning artefacts have any homology to known nucleotide or amino acid sequences, as shown by BLASTX and BLASTN searches.

D6(14)B, consisting of about 100 nucleotides, is upregulated in secretion medium compared to growth medium, as shown in the Southern blot using cDNA from cells grown in growth medium and in secretion medium (Figure 6f). We have 56 nucleotides of sequence from the T7 primer and 25 bases from the SP6 primer. These sequences are both novel. The T7 sequence bears a resemblance to a peptide derived from a rat D(2) dopamine receptor, revealed by a BLASTX search, suggesting that the candidate may be a G-protein coupled receptor (see appendix). Again, it is impossible to know the significance of this until a full length clone has been identified and it is established whether this is actually the appropriate reading frame. The SP6 sequence does not show any high-probability homology at the nucleotide or amino acid level with any known sequences, as shown in the BLASTN and BLASTX searches.

### Conclusions

1. The subcellular targeting of GLUT1, the only glucose transporter isoform expressed in mammary gland, is specifically regulated by prolactin and dexamethasone in order to support the metabolic demands of lactation, which include the need for enhanced glucose transport across the basal membrane and into the Golgi of mammary epithelial cells. This is shown schematically in Figure 7. The data included in this progress report demonstrate unambiguously, by independent methods and both in an established cell line and in the lactating murine gland, that GLUT1 is localized in the Golgi in response to the hormonal milieu of lactation; corresponding with this, lactose biosynthesis is enhanced several-fold. A manuscript including these data is being prepared for submission. This conclusion strengthens the rationale of searching for the proteins responsible for this unique targeting, and offers the possibility that once this mechanism is understood, it might be manipulated to alter glucose transport in neoplastic mammary epithelial cells.
2. Developmental regulation of glucose transporter isoforms in mammary gland is isoform specific. GLUT1 mRNA and protein, but not GLUT5 mRNA and protein, are increased during lactation compared to virgin tissue. Upon weaning for 24 hours, GLUT1 mRNA levels do not change, but GLUT1 protein levels fall significantly. This suggests that although transcriptional control may be important for the induction of GLUT1 during lactation, that mechanisms involving translational efficiency, protein degradation, or both are more important at the time of weaning. Once this phenomenon is understood mechanistically, this may offer the basis for another opportunity to alter glucose transport in neoplastic mammary epithelial cells.
3. Six genes have been identified as differentially expressed in response to prolactin and dexamethasone in CIT3 cells, and thus as potential candidates to account for the altered glucose transporter targeting seen in secretion medium. One of these is LDH-A, and five are novel. Potential homology of certain candidates to GLUT4, NADH dehydrogenase, guanylate cyclase, and G-protein coupled receptors, has been noted, but it is impossible to know the significance of these potential homologies until full length clones have been identified and it is established whether potential homologies correspond to appropriate reading frames.

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AppendicesFigure Legends

Figure 1. Colocalization of GLUT1 and the Golgi marker Bodipy-TR in CIT3 cells exposed to secretion medium but not growth medium. Cells were treated as described in Experimental Methods. Green indicates fluorescent antibody staining of GLUT1. Red indicates the Golgi marker Bodipy TR. Yellow indicates colocalization of the two signals. Note that this is seen in secretion medium, while distinct red and green signals are seen in growth medium. This indicates that GLUT1 is found in the Golgi in secretion medium but not in growth medium. (Note that this figure can not be presented in black and white.)

Figure 2. Subcellular fractionation of GLUT1 by iodixanol density gradient in CIT3 cells. Fractionation was carried out as described in Experimental Methods. Each lane represents 20 mcg of protein. The upper panel shows cells exposed to growth medium, the lower panel cells exposed to secretion medium. Note that GLUT1 is induced in secretion medium, consistent with previous data, and that fractions 1-3, the least dense fractions of a 15% iodixanol gradient, corresponding to Golgi, are highly enriched in GLUT1 in cells grown in secretion medium but not in growth medium. Hom, homogenate; Nuc, nuclear fraction; 3K SN, supernatant of 3000g centrifugation; Mito, mitochondrial fraction; microsomal, microsomal fraction; 100K SN, supernatant of 100,000g centrifugation; fractions 1-3, least dense fractions of density gradient; GLUT1, standard.

Figure 3. Subcellular fractionation of GLUT1 by iodixanol density gradient in mammary gland of mice on day 17 of lactation. Fractionation was carried out as described in Experimental Methods. Each lane represents 20 mcg of protein. The upper panel shows gland from an actively lactating mother, the lower panel gland from a mother whose pups were removed 24 hours earlier. Note that GLUT1 is abundant in lactating gland and barely detectable in gland from the 24-hour weaned mother. Also note that fractions 1-2, the least dense fractions of a 15% iodixanol gradient, corresponding to Golgi, are highly enriched in GLUT1 in actively lactating gland, but that GLUT1 is absent from Golgi of the weaned mother. Hom, homogenate; Nuc, nuclear fraction; 3K SN, supernatant of 3000g centrifugation; Mito, mitochondrial fraction; microsomal, microsomal fraction; 100K SN, supernatant of 100,000g centrifugation; fractions 1-2, least dense fractions of density gradient; GLUT1, standard.

Table 1. Lactose levels in tissue culture medium. Lactose assay was carried out as described in Experimental Methods. Results are from numerous attempts using different conditions and durations of incubation.

Table 2. Lactose biosynthesis in CIT3 cells in growth medium and in secretion medium. Lactose biosynthesis was measured as described in Experimental Methods. Note that the data are crude in that lactose was not purified by TLC, and therefore reflect upper limits of lactose biosynthesis. Results are means of three determinations, with a standard error of less than 10% for all values.

Figure 4. Northern blot of GLUT1 mRNA in mammary gland and CIT3 cells. The blot was prepared as described in Experimental Methods. Two samples each are included for 8 week old virgins, and mothers who had been lactating for 10 or 17 days; one sample each is included for a mother who had been lactating for 9 days, then weaned for 1 day, for CIT3 cells grown in growth and secretion medium, and for adult mouse brain and liver. Note that virgin gland expresses little GLUT1, and that weaning has little effect on GLUT1 mRNA. Also note that secretion medium has little effect compared to growth medium.

Figure 5. Northern blot of GLUT5 mRNA in mammary gland and CIT3 cells. The blot was prepared as described in Experimental Methods. Two samples each are included for 8 week old virgins, and mothers who had been lactating for 10 or 17 days; one sample each is included for a mother who had been lactating for 9 days, then weaned for 1 day, for CIT3 cells grown in growth and secretion medium, and for adult mouse brain and liver. Note that very little GLUT5 is detected in mammary gland at any stage. Also note significant levels of GLUT5 mRNA in CIT3 cells.

Figure 6. Differential expression of six genes in response to prolactin and dexamethasone: a) D6(5)H, b) D6(5)#8, c) D6(9), d) D6(14)A, e) D9(14)#63, and f) D6(14)B. Genes are named according to the primer combination of the PCR reaction that led to their identification, and by an additional number or letter if needed to distinguish more than one gene arising from the same primer pair. Probes were prepared and blots performed as described in Experimental Methods. Probes included, for a) and b), RNA probes prepared by in vitro transcription of PCR products, and for d), e), and f), cDNA from cells grown in growth medium and secretion medium; for c), the original DD-PCR reactions are shown.

Figure 7. Diagram of the regulation of glucose transporter amount and subcellular targeting in CIT3 cells. Note that secretion medium results in an increase in the amount of GLUT1, in the polarization of GLUT1 targeting with preference for basal and not apical membrane, and in the sequestration of GLUT1 within the Golgi. This is based on evidence presented in the text based on work done during the first two years of this grant.

Genbank search summaries

- a) D6(5)H  
BLASTN, BLASTX, GLUT4 homology
- b) D6(5)#8  
BLASTN, BLASTX
- c) D6(9)  
BLASTN, BLASTX
- d) D6(14)A  
BLASTN, BLASTX
- e) D9(14)#63  
BLASTN, BLASTX
- f) D6(14)B  
BLASTN, BLASTX

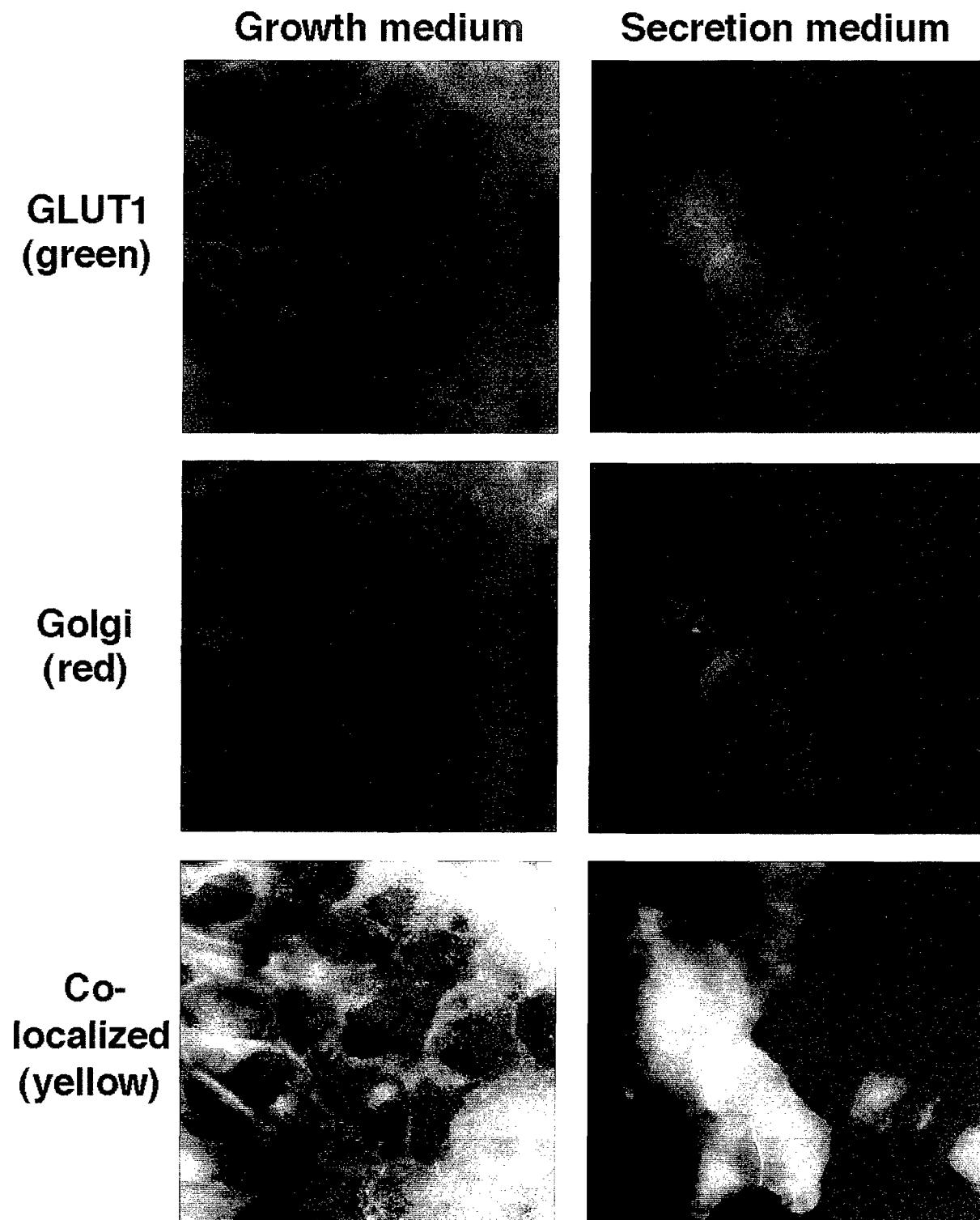


Figure 1

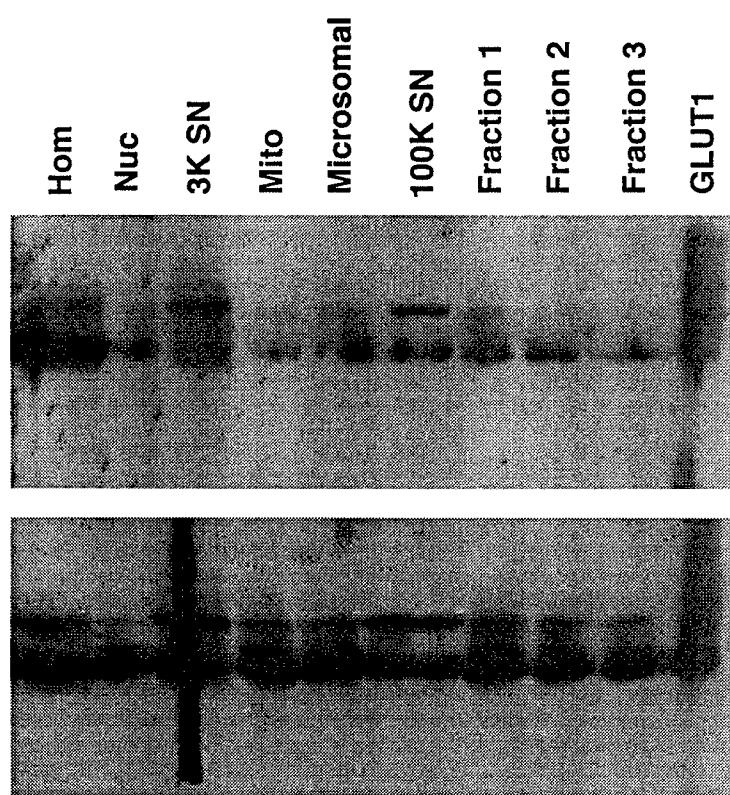


Figure 2

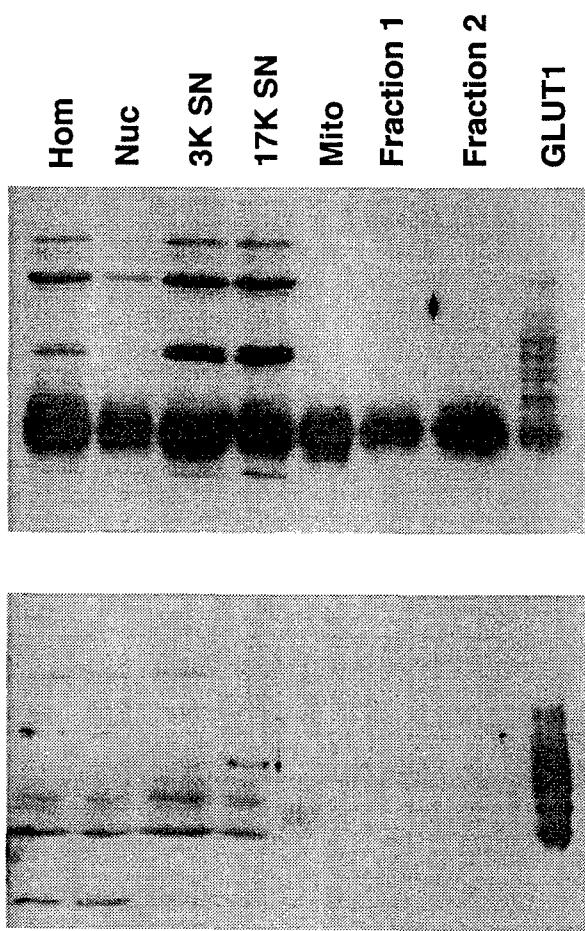


Figure 3

Table 1. Lactose levels in tissue culture medium

		Lactose
	plastic	Matrigel
CIT3- Growth medium	<5 mg/l	<5 mg/l
CIT3- Secretion medium	<5 mg/l	<5 mg/l
Primary cells, mouse mammary gland	<5 mg/l	<5 mg/l
Primary cells, human milk	<5 mg/l	<5 mg/l

Table 2. Lactose biosynthesis

	Lactose synthesis (cpm/mg protein)	
	homogenate	Golgi
CIT3- Growth medium	2848	218
CIT3- Secretion medium	4641	1073

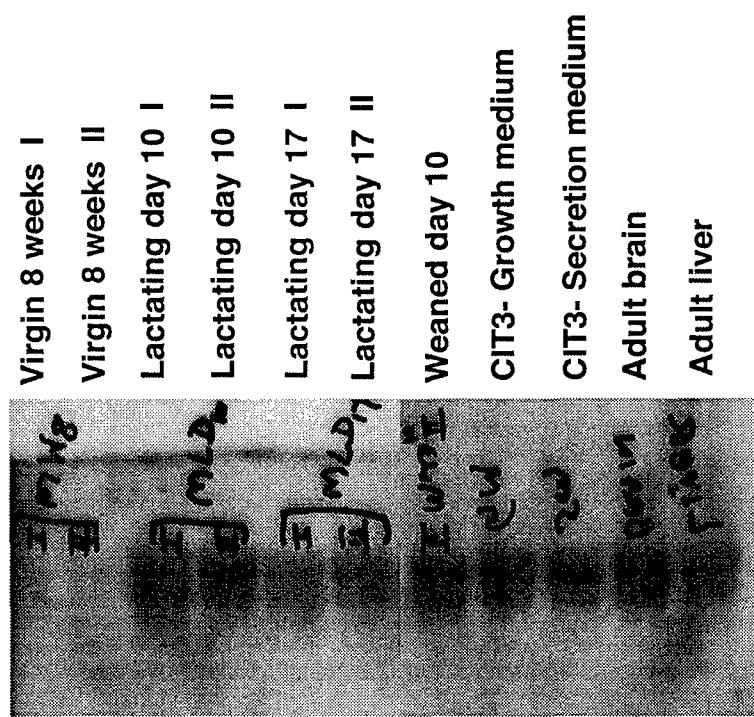


Figure 4

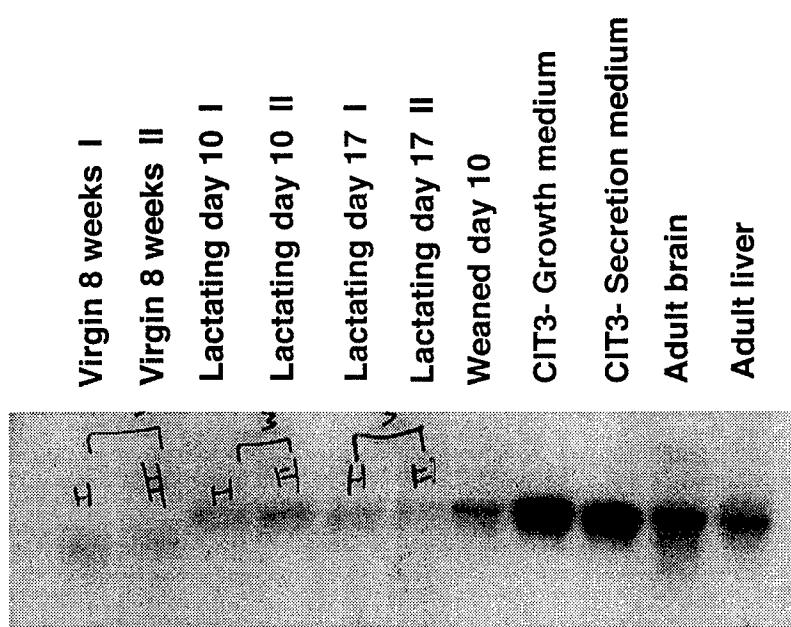


Figure 5

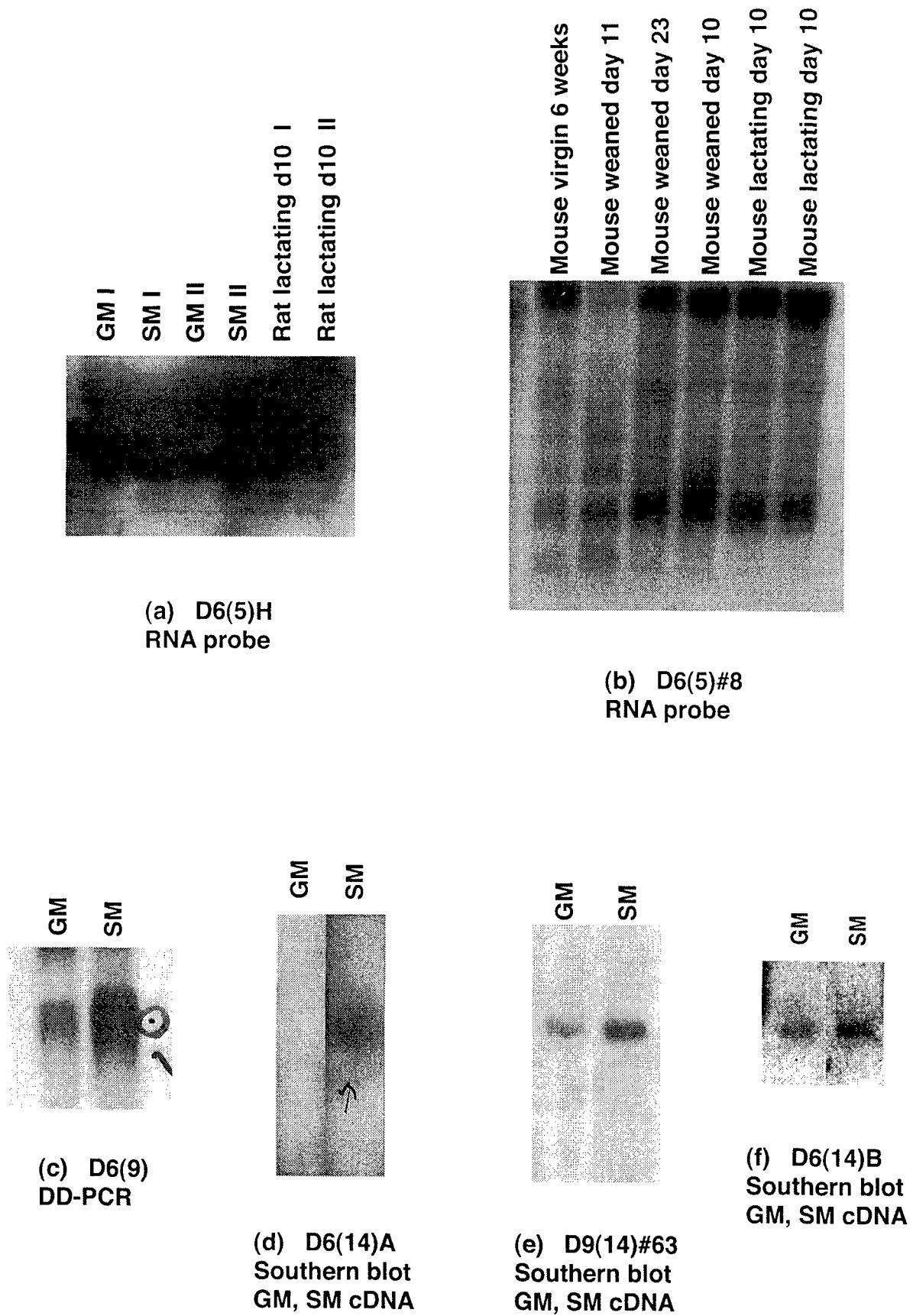


Figure 6

**Query=** CLONE H ACAGTGAGGGAATGAGGTTGACTAATGCATAT  
(32 letters)

**Database:** Non-redundant GenBank+EMBL+DDBJ+PDB sequences  
272,299 sequences; 376,297,050 total letters.

Sequences producing High-scoring Segment Pairs:		High Score	Probabil P(N)	Smalles Sum
<u>emb X72974 HSPKCIE</u>	H.sapiens gene for protein kinase C i...	91	0.996	
<u>gb J02823 CHKMLCL23</u>	Chicken myosin light chain L23 mRNA, ...	90	0.9997	
<u>gb M34990 CHKMLCE2</u>	Chicken embryonic myosin alkali light...	90	0.9998	
<u>emb X14429 GDMYAL37</u>	G.domesticus DNA for embryonic myosin...	90	0.9998	

**emb|X72974|HSPKCIE** *H.sapiens* gene for protein kinase C isoform epsilon  
Length = 218

### Plus Strand HSPs:

Score = 91 (25.1 bits), Expect = 5.5, P = 1.0  
Identities = 23/29 (79%), Positives = 23/29 (79%), Strand = Plus / Plus

Query: 4 GTGAGGGAAATGAGGTTGACTAATGCATAT 32  
          ||||||| ||||| ||| ||| ||| |||  
Sbjct: 31 GTGAGGGAAATGAGTATGAACAAAGGCACAT 59

**pir|A19197|A19197** class II histocompatibility antigen HLA-DR beta chain -  
human (fragment)  
Length = 34

### Minus Strand HSPs:

Score = 26 (12.0 bits), Expect = 1.5, Sum P(2) = 0.77  
Identities = 5/8 (62%), Positives = 6/8 (75%). Frame = -2

uery: 30 LLQLITYN 7  
bjct: 25 LLELFIYN 32

Score = 19 (8.7 bits), Expect = 1.5, Sum P(2) = 0.77

### GLUT4 Glucose transporter

Query: SVTP LL QLITYNTN  
GLUT4: 467 TP LL Q RGRTFDQISAAFHRTPSLLEQEVKPSTELEYLGPDEND 509

**Query=** D6 (5) #8 TGGTNGTAGATTGCGAAATGTATTCAGGGAAAATCGAAGAGATAA  
(253 letters)

**Database:** Non-redundant GenBank+EMBL+DDBJ+PDB sequences  
272,299 sequences; 376,297,050 total letters.

Sequences producing High-scoring Segment Pairs:				High Score	Smallest Sum Probabil P(N)
gb U50834 CRU50834	Cowdria ruminantium Nyatsanga major ...	120	0.97		
gb U50831 CRU50831	Cowdria ruminantium Crystal Springs ...	120	0.97		
gb U50833 CRU50833	Cowdria ruminantium Highway major an...	120	0.97		
gb U49843 CRU49843	Cowdria ruminantium major antigenic ...	120	0.97		
emb Z68161 CEF20C5	Caenorhabditis elegans cosmid F20C5	120	0.98		
emb Z70206 CEC49F8	Caenorhabditis elegans cosmid C49F8	119	0.99		
emb Z75213 SCYOR305W	S.cerevisiae chromosome XV reading f...	117	0.999		
emb Z75212 SCYOR304W	S.cerevisiae chromosome XV reading f...	117	0.999		
gb L23504 ALFPRP4ENO	Medicago truncatula early nodulin (p...	116	0.9997		

**gb|U50834|CRU50834** *Cowdria ruminantium* Nyatsanga major antigenic protein 1 (map1) gene, complete cds.  
Length = 1101

### Plus Strand HSPs:

Score = 120 (33.2 bits), Expect = 3.6, P = 0.97  
Identities = 40/60 (66%). Positives = 40/60 (66%). Strand = Plus / Plus

**Query=** D6(5) #8 TGGTNGTAGATTGCGAAATGTATTCACGGAAAATCGAAGAGATAA  
(253 letters)

Translating both strands of query sequence in all 6 reading frames

**Database:** Non-redundant GenBank CDS  
translations+PDB+SwissProt+SPupdate+PIR  
225,697 sequences; 63,534,404 total letters.

		Reading Frame	High Score	Smalles Sum	Probabil P(N)
Sequences producing High-scoring Segment Pairs:					
<u>pir  s34500</u>	hypothetical protein 241 (psbC 3'... -2	<u>36</u>	0.021		
<u>gi 1335774</u>	(U27466) IAP-like protein [Gallus... -1	<u>38</u>	0.033		
<u>pir B34751</u>	hypothetical protein - African ma... -1	<u>39</u>	0.062		
<u>pir I30010</u>	NADH dehydrogenase (ubiquinone) (...) -1	<u>38</u>	0.069		
<u>prf 1101341C</u>	HURF 5 [Leishmania tarentolae] -1	<u>38</u>	0.069		
<u>prf 1306334B</u>	dehydrogenase 5, NADH [Leishmania ... -1	<u>38</u>	0.095		
<u>sp P27286 VGLF PI2HG</u>	FUSION GLYCOPROTEIN PRECURSOR (CO... -3	<u>34</u>	0.14		
<u>sp P25467 VGLF PI2H</u>	FUSION GLYCOPROTEIN PRECURSOR (CO... -3	<u>34</u>	0.14		
<u>sp P26629 VGLF PI2HT</u>	FUSION GLYCOPROTEIN PRECURSOR (CO... -3	<u>34</u>	0.14		
<u>gnl PID e244963</u>	(Z73898) ZK822.4 [Caenorhabditis ... +3	<u>36</u>	0.16		
<u>sp P31916 YCX3_EUGGR</u>	HYPOTHETICAL 78.0 KD PROTEIN IN P... -2	<u>36</u>	0.19		
<u>gnl PID e252056</u>	(Z75083) ORF YOR175c [Saccharomy... -3	<u>37</u>	0.23		
<u>gi 1280087</u>	(U55369) similar to human multidr... -2	<u>36</u>	0.50		
<u>gi 1244785</u>	(U43703) Tbf1p [Saccharomyces cer... -2	<u>33</u>	0.50		
<u>gnl PID e217966</u>	(Z68493) C33A12.6 [Caenorhabditis... -2	<u>46</u>	0.65		
<u>gi 1223896</u>	(U42425) DmMstProx [Drosophila me... -2	<u>35</u>	0.66		
<u>gi 1072088</u>	(U37270) polymerase polyprotein [...] +2	<u>35</u>	0.66		
<u>sp P48257 YCF5_CYAPA</u>	PROBABLE CYTOCHROME C BIOGENEGIS ... -1	<u>34</u>	0.77		
<u>sp P01513 ATTE_HYACE</u>	ATTACIN E AND F PRECURSOR (IMMUNE... -2	<u>48</u>	0.98		

pir||s34500 hypothetical protein 241 (psbC 3' region) - Euglena

gracilis chloroplast  
Length = 241

Minus Strand HSPs:

Score = 36 (16.6 bits), Expect = 0.021, Sum P(3) = 0.021  
Identities = 8/21 (38%), Positives = 12/21 (57%), Frame = -2

Query: 96 LGFISTNSHTPLVSTQLYLFD 34  
LGFI P+ + +L LF+  
Sbjct: 117 LGFIHPFKFRPIGNVRLLLFE 137

Score = 33 (15.2 bits), Expect = 0.021, Sum P(3) = 0.021  
Identities = 9/25 (36%), Positives = 12/25 (48%), Frame = -3

Query: 248 LFVCLFVFYVKHF\*IKNYFPHVN 174  
L+ CLF F + NY+ V F  
Sbjct: 37 LYNCLFEFKHLVLLYNYSPVYSF 61

Score = 30 (13.8 bits), Expect = 0.021, Sum P(3) = 0.021  
Identities = 6/18 (33%), Positives = 11/18 (61%), Frame = -3

Query: 137 LYAFFFKETHVKII\*DSY 84  
+Y+FF K K+ +S+  
Sbjct: 58 VYSFFSKRQRYKLN 75

pir||130010 NADH dehydrogenase (ubiquinone) (EC 1.6.5.3) chain 5 -  
Sauroleishmania tarentolae mitochondrion (SGC6)  
Length = 502

Minus Strand HSPs:

Score = 38 (17.5 bits), Expect = 0.071, Sum P(3) = 0.069  
Identities = 7/9 (77%), Positives = 8/9 (88%), Frame = -1

Query: 247 FLFVCLFFM 221  
FL VCLFF+  
Sbjct: 423 FLCVCLFFI 431

Score = 35 (16.1 bits), Expect = 0.17, Sum P(3) = 0.16  
Identities = 5/14 (35%), Positives = 8/14 (57%), Frame = -3

Query: 251 FLFVCLFVFYVKHF 210  
F F+ + FY +F  
Sbjct: 105 FCFIVFYAFYYMYF 118

Score = 33 (15.2 bits), Expect = 0.53, Sum P(3) = 0.41  
Identities = 6/10 (60%), Positives = 7/10 (70%), Frame = -3

Query: 251 FLFVCLFVFY 222  
FL VCLF +  
Sbjct: 423 FLCVCLFFIF 432

Score = 32 (14.7 bits), Expect = 0.071, Sum P(3) = 0.069  
Identities = 7/11 (63%), Positives = 7/11 (63%), Frame = -2

Query: 63 LVSTQLYLFDF 31  
LV T LFDF  
Sbjct: 454 LVDTLFLLFDF 464

Score = 32 (14.7 bits), Expect = 6.8, Sum P(3) = 1.0  
Identities = 11/41 (26%), Positives = 15/41 (36%), Frame = -1

Query: 241 FVCLFFMLNIFR\*KIISLMSMFLRMRLFEIFNTFYTLFFL 119  
F C +L + + FL + L F F Y F L  
Sbjct: 402 FYCKDLLLCLLMLTSFFFFILEFLCVCLFFIFFTVIYNYFLL 442

Score = 31 (14.3 bits), Expect = 0.071, Sum P(3) = 0.069  
Identities = 5/13 (38%), Positives = 8/13 (61%), Frame = -1

Query: 157 FEIFNTFYTLFFL 119  
F + ++ LFFL  
Sbjct: 433 FTVIYNYFLLFFL 445

Score = 30 (13.8 bits), Expect = 6.8, Sum P(3) = 1.0  
Identities = 5/9 (55%), Positives = 6/9 (66%), Frame = -3

Query: 248 LFVCLFVFY 222  
LF C +FY  
Sbjct: 339 LFFCYHMFY 347

**Query=** D6 (9) GGTATAACATAAAACACAGTTTATTTCGGAAATTCA CONTAINS UNPUBLISHED DATA  
(36 letters)

**Database:** Non-redundant GenBank+EMBL+DDBJ+PDB sequences  
272,299 sequences; 376,297,050 total letters.

Sequences producing High-scoring Segment Pairs:				High Score	Probabil P(N)	Smalles Sum
<u>gb M22562 RATGCS</u>	Rat soluble guanylate cyclase 70kd su...	<u>107</u>	0.28			
<u>emb Z47356 CET15H9</u>	Caenorhabditis elegans cosmid T15H9	<u>105</u>	0.38			
<u>emb X06534 ECP307OT</u>	E. coli plasmid Ent P307 oriT region	<u>103</u>	0.47			
<u>emb X73832 HISODA</u>	H.influenzae DNA for type b manganese...	<u>103</u>	0.49			
<u>gb M62986 P30TRA</u>	Plasmid P307 p19, traM, traJ, traY ge...	<u>103</u>	0.50			
<u>gb U32789 HIU32789</u>	Haemophilus influenzae from bases 115...	<u>103</u>	0.50			
<u>emb X55896 ECPSU233</u>	E.coli plasmid pSU233 oriT, traM, fin...	<u>99</u>	0.77			
<u>emb Z68302 CEZK792</u>	Caenorhabditis elegans cosmid ZK792	<u>98</u>	0.84			
<u>gb U09343 MDU09343</u>	Musca domestica sbo cytochrome P450 (...)	<u>97</u>	0.89			
<u>gb L26498 ATHFUS6A</u>	Arabidopsis thaliana (FUS6) gene, com...	<u>95</u>	0.96			
<u>gb U22833 CELW02B3</u>	Caenorhabditis elegans cosmid W02B3.	<u>95</u>	0.96			
<u>emb Z79253 HSPA26H8</u>	H.sapiens flow-sorted chromosome 6 Ta...	<u>94</u>	0.97			
<u>emb Z79598 CEC44H4</u>	Caenorhabditis elegans cosmid C44H4	<u>80</u>	0.99			
<u>gb U11253 ATU11253</u>	Arabidopsis thaliana Columbia ecotype...	<u>92</u>	0.997			
<u>gb U11255 ATU11255</u>	Arabidopsis thaliana Columbia ecotype...	<u>92</u>	0.997			
<u>gb U39647 CELC44E12</u>	Caenorhabditis elegans cosmid C44E12	<u>92</u>	0.997			
<u>gb U46670 CELR09G11</u>	Caenorhabditis elegans cosmid R09G11.	<u>92</u>	0.997			
<u>emb X03228 ECEBGRA</u>	E. coli ebgR and ebgA genes for ebg r...	<u>90</u>	0.9998			
<u>emb X52031 ECEBG</u>	Escherichia coli wildtype ebg operon ...	<u>90</u>	0.9998			
<u>emb Z75549 CET19C4</u>	Caenorhabditis elegans cosmid T19C4	<u>90</u>	0.9998			
<u>gb U18997 ECOUW67</u>	Escherichia coli K-12 chromosomal req...	<u>90</u>	0.9998			

gb|M22562|RATGCS Rat soluble guanylate cyclase 70kd subunit mRNA,  
complete cds.  
Length = 3047

### Plus Strand HSPs:

Score = 107 (29.6 bits), Expect = 0.32, P = 0.28  
Identities = 23/25 (92%), Positives = 23/25 (92%), Strand = Plus / Plus

Query: 3 TATAACATAAAACACAGTTTATTTC 27  
| | | | | | | | | | | | | | | | | | | |  
Sbct: 2348 TATAACATAAAACAAAGTTTATATC 237

**Query=** D6(9) GGTATAACATAAAACACAGTTATTCGGAAATTCA  
(36 letters)

Translating both strands of query sequence in all 6 reading frames

**Database:** Non-redundant GenBank CDS  
translations+PDB+SwissProt+SPupdate+PIR  
225,697 sequences; 63,534,404 total letters.

Sequences producing High-scoring Segment Pairs:	Reading Frame	High Score	Smalles Sum Probabil P(N)
<u>gi 805315</u> heat shock protein-specific T-cell receptor ...	+1	23	0.990

gi|805315 heat shock protein-specific T-cell receptor beta chain VDJ region {clone MV17} [human, peripheral blood mononuclear cells, Peptide Partial, 29 aa]  
Length = 29

Plus Strand HSPs:

Score = 23 (10.6 bits), Expect = 4.6, Sum P(2) = 0.99  
Identities = 4/6 (66%), Positives = 5/6 (83%), Frame = +1

Query: 13 NTVYFG 30  
+T YFG  
Sbjct: 16 DTQYFG 21

Score = 20 (9.2 bits), Expect = 4.6, Sum P(2) = 0.99  
Identities = 3/4 (75%), Positives = 4/4 (100%), Frame = +3

Query: 3 YNIK 14  
YN+K  
Sbjct: 5 YNLK 8

**Query=** #6 PAMP10 GGAACCAATCACCCAAAGTGTATGCCAAATAAAACCTTGAACAG  
(44 letters)

**Database:** Non-redundant GenBank+EMBL+DDBJ+PDB sequences  
272,299 sequences; 376,297,050 total letters.

Sequences producing High-scoring Segment Pairs:				High Score	Probabil P(N)	Smalles Sum
<u>emb</u>   X02526   MMA4LDH7	M.musculus	LDH-A gene 3' region for 1...		<u>211</u>	6.4e-10	
<u>gb</u>   M17516   MUSLDH8B	Mouse	lactate dehydrogenase A-4 (LDH-...		<u>211</u>	6.4e-10	
<u>gb</u>   U13687   MMU13687	Mus musculus	DBA/2J lactate dehydroge...		<u>211</u>	6.8e-10	
<u>emb</u>   Y00309   MMLDHAG	Mouse	LDH-A gene for lactate dehydrou...		<u>211</u>	7.3e-10	
<u>emb</u>   X02153   HSLDHAPG	Human	pseudogene for lactate dehydrog...		<u>126</u>	0.00011	
<u>gb</u>   M15732   MUSLDHAPS	Mouse	lactate dehydrogenase-A (LDH-A)...		<u>143</u>	0.00032	
<u>gb</u>   M54926   RATLADA	Rat	lactate dehydrogenase A mRNA, 3' ...		<u>139</u>	0.00064	
<u>emb</u>   X01964   RNLADEH1	Rat	mRNA for lactate dehydrogenase		<u>139</u>	0.00068	
<u>emb</u>   X05163   MMLDHAP1	Mouse	lacatate dehydrogenase-A (LDH-A...		<u>139</u>	0.00069	
<u>gb</u>   I23884   I23884	Sequence	3 from patent US 5539096		<u>135</u>	0.00081	
<u>gb</u>   M17518   MUSLDHPC	Mouse	lactate dehydrogenase A-4 pseud...		<u>121</u>	0.021	
<u>emb</u>   X05164   MMLDHAP2	Mouse	lactate dehydrogenase-A (LDH-A)...		<u>103</u>	0.50	
<u>gb</u>   S79912   S79912	GHRH-R=growth	hormone-releasing hormo...		<u>99</u>	0.75	
<u>gb</u>   U17579   HSU17579	Human	growth hormone-releasing hormon...		<u>99</u>	0.77	
<u>emb</u>   X00601   DDRN05	Dictyostelium	discoideum ribosomal RN...		<u>98</u>	0.84	
<u>gb</u>   M63704   RICCYTC	Rice	cytochrome c (OsCc-1) gene, comp...		<u>96</u>	0.93	
<u>emb</u>   X80301   NTAXI1G	N.tabacum	axi 1 gene		<u>95</u>	0.96	
<u>emb</u>   X12727   AOAMY3	Aspergillus	oryzae amy3 gene for alph...		<u>94</u>	0.98	
<u>emb</u>   X56838   HSGSTMU3	Human	GSTMu3 gene for a glutathione S...		<u>92</u>	0.996	
<u>emb</u>   Z66567   CEZK455	Caenorhabditis	elegans cosmid ZK455		<u>92</u>	0.997	
<u>emb</u>   X57698   ATACPA1	A.thaliana	DNA for acyl carrier prote...		<u>72</u>	0.998	
<u>emb</u>   X61082   VARRNINT	V.angustifolia	25S/18S rRNA intergeni...		<u>90</u>	0.9998	

emb|X02526|MMA4LDH7 *M.musculus* LDH-A gene 3' region for lactate dehydrogenase A4 isoenzyme  
Length = 863

### Plus Strand HSPs:

Score = 211 (58.3 bits), Expect = 6.4e-10, P = 6.4e-10  
Identities = 43/44 (97%), Positives = 43/44 (97%), Strand = Plus / Plus

Query: 1 GGAACCAATCACCCAAAGTGTATGCCAATAAAACCTTGAACAG 44

Sbjct: 614 GGAACCAACCACCCAAAGTGTCAATGCCAAATAAACCTTGAACAG 657

**Query=** D(9)14 #63 T7 (SEE ALSO SP6)  
 CCCACATGTAACCAAAAAAAAAAAAAACCAACAAACNAAAACTGGAGCTGGAACCAATCGAG  
 CCAAAAAAAAAAGCCAAAAACGAAAACGAGCTGGAGCATGGAACCAAAT  
 (124 letters)

**Database:** Non-redundant GenBank+EMBL+DDBJ+PDB sequences  
 272,299 sequences; 376,297,050 total letters.

Sequences producing High-scoring Segment Pairs:	Smalles	Sum	High	Probabil
	Score			P(N)

Parameters:

V=100  
 B=50  
 H=0  
 P=4

**Query=** D(9)14 #63 T7 (SEE ALSO SP6)  
 CCCACATGTAACCAAAAAAAAAAAAAACCAACAAACNAAAACTGGAGCTGGAACCAATCGAG  
 CCAAAAAAAAAAGCCAAAAACGAAAACGAGCTGGAGCATGGAACCAAAT  
 (124 letters)

Translating both strands of query sequence in all 6 reading frames

**Database:** Non-redundant Database of GenBank EST Division  
 675,150 sequences; 244,264,523 total letters.

Sequences producing High-scoring Segment Pairs:	Smalles	Sum	Reading	High	Probabil
	Frame		Score		P(N)

Parameters:

V=100  
 B=50  
 H=0  
 -filter=SEG  
 P=4

**Query=** D(9)14 #63 SP6 (SEE ALSO T7)  
 TAGCTCGATTGGTCCAGTTTTGTTGGTTTTTTGGTTGGTTTTTTGGGGGGTTGGTTTT  
 (62 letters)

**Database:** Non-redundant GenBank+EMBL+DDBJ+PDB sequences  
 272,299 sequences; 376,297,050 total letters.

	Smalles	Sum	High	Probabil
Score				P(N)

Sequences producing High-scoring Segment Pairs:

Parameters:

V=100  
 B=50  
 H=0  
 P=4

**Query=** D(9)14 #63 SP6 (SEE ALSO T7)  
 TAGCTCGATTGGTCCAGTTTTGTTGGTTTTTTGGTTGGTTTTTTGGGGGGTTGGTTTT  
 (62 letters)

Translating both strands of query sequence in all 6 reading frames

**Database:** Non-redundant Database of GenBank EST Division  
 675,150 sequences; 244,264,523 total letters.

	Smalles	Sum	High	Probabil
Reading Frame				P(N)

Sequences producing High-scoring Segment Pairs:

Parameters:

V=100  
 B=50  
 H=0  
 -filter=SEG  
 P=4

Query= D6(14)B (NOT LDH) T7 (SEE ALSO SP6) CONTAINS UNPUBLISHED DATA  
 ATACATTTTCATTTATTCTTCCTCCTTAGCTTGGGGATTAAACCAATCGG  
 (56 letters)

Database: Non-redundant GenBank+EMBL+DDBJ+PDB sequences  
 272,299 sequences; 376,297,050 total letters.

Sequences producing High-scoring Segment Pairs:		High Score	P(N)	Smallest Sum Probability
gb U39744 CELC03F11	Caenorhabditis elegans cosmid C03F11.	109	0.20	1
emb X74206 LLCPYRDA	L.lactis gene for dihydroorotate deh...	107	0.27	1
gb U13642 CELZC395	Caenorhabditis elegans cosmid ZC395	82	0.32	2
emb Z24725 HSMIG2GNA	H.sapiens mitogen inducible gene mig...	106	0.32	1
gb U32789 HIU32789	Haemophilus influenzae from bases 11...	106	0.33	1
emb X69019 MMHOX35MR	M.musculus Hox-3.5 mRNA	105	0.37	1
emb Z70265 CEC05A9	Caenorhabditis elegans cosmid C05A9	104	0.44	1
emb Z15058 ATBCBG	A.thaliana of bcb gene encoding blue...	103	0.50	1
emb X66379 XLLIPIG	X.luminescens LipI gene for lipase	103	0.50	1
emb X52030 RSIID5G	Rat P450 IID5 gene.	103	0.50	1
gb L06178 AMFGENOM	Apis mellifera mitochondrial genome.	87	0.62	2
emb Z80362 HSDRBPE	H.sapiens HLA-DRB pseudogene, exon 1;	102	0.65	1
dbj D49525 CELTPA1A	Caenorhabditis elegans gene for TPA...	102	0.65	1
emb Z68105 CEF13E6	Caenorhabditis elegans cosmid F13E6	102	0.65	1
emb X87344 HSEVMHC	H.sapiens DMA, DMB, HLA-Z1, IPP2, LM...	87	0.80	2
gb U35016 MHU35016	Mycoplasma hyorhinis VlpD prolipopro...	101	0.84	1
emb Z30215 CEF40F12	Caenorhabditis elegans cosmid F40F12	101	0.84	1
dbj D85922 HUMQ16F8	Human DNA for single-minded gene 2, ...	101	0.84	1
gb L31759 HUMUT5141B	Human STS UT5141, 3' primer bind.	100	0.91	1
emb X55124 PFRESA	P.falciparum DNA fragment for ring-i...	100	0.93	1
gb U55885 HSPON1EX9	Human paraoxonase (PON1) gene, exon ...	74	0.94	2
gb L34881 YSPPP2A	Schizosaccharomyces pombe protein ph...	100	0.94	1
emb X05181 PFRESAR2	P.falciparum NF7 Ag 13 RESA mRNA for...	100	0.94	1
gb L20009 SIVP209C15	Simian immunodeficiency virus (clone...	100	0.94	1
gb L20008 SIVP209C19	Simian immunodeficiency virus type 1...	100	0.94	1
emb X57025 HSIGFACI	Human IGF-I mRNA for insulin-like gr...	74	0.94	2
gb L20099 SIVP209C14	Simian immunodeficiency virus envelo...	100	0.94	1
gb M22222 YSCREV1	Yeast (S.cerevisiae) REV1 gene requi...	100	0.94	1

gb|U39744|CELC03F11 Caenorhabditis elegans cosmid C03F11.  
 Length = 30,601

Plus Strand HSPs:

Score = 109 (30.1 bits), Expect = 0.22, P = 0.20  
 Identities = 25/29 (86%), Positives = 25/29 (86%), Strand = Plus / Plus

Query: 8 TTCATTTATTCTTCCTCCTTAGCTTG 36  
 ||||| ||| ||||| ||| ||||| ||| |||||  
 Sbjct: 29228 TTCACTTTCTCTTCTTAGCTTG 29256

Query= D6(14)B (NOT LDH) T7 (SEE ALSO SP6)  
 ATACATTTTCATTTATTCTTCCTTAGCTTGGGGATTAAACCAAATCGG  
 (56 letters)

Translating both strands of query sequence in all 6 reading frames

Database: Non-redundant GenBank CDS  
 translations+PDB+SwissProt+SPupdate+PIR  
 225,697 sequences; 63,534,404 total letters.

Sequences producing High-scoring Segment Pairs:	Reading Frame	High Score	Smallest Sum Probability		N
			P(N)	Probability	
pat US 5508384 144 Sequence 144 from patent US 5508384	+1	30	0.39	2	
pat US 5508384 184 Sequence 184 from patent US 5508384	+1	29	0.49	2	
pat US 5508384 183 Sequence 183 from patent US 5508384	+1	29	0.72	2	
pat US 5508384 219 Sequence 219 from patent US 5508384	+1	29	0.72	2	
pat US 5508384 221 Sequence 221 from patent US 5508384	+1	24	0.96	2	
pat US 5508384 181 Sequence 181 from patent US 5508384	+1	26	0.98	2	
pat US 5508384 113 Sequence 113 from patent US 5508384	+1	23	0.99	2	
pat US 5508384 121 Sequence 121 from patent US 5508384	+1	23	0.99	2	
gi 485356 (M76696) precore protein [Hepatitis...	+3	22	0.998	2	

pat|US|5508384|144 Sequence 144 from patent US 5508384  
 Length = 35

Plus Strand HSPs:

Score = 30 (13.8 bits), Expect = 0.49, Sum P(2) = 0.39  
 Identities = 7/10 (70%), Positives = 8/10 (80%), Frame = +1

Query: 19 LSFLSLGDFN 48  
 LSFLSL F+  
 Sbjct: 12 LSFLSLSVFS 21

Score = 20 (9.2 bits), Expect = 0.49, Sum P(2) = 0.39  
 Identities = 3/5 (60%), Positives = 5/5 (100%), Frame = +2

Query: 5 IFILF 19  
 IF+L+  
 Sbjct: 3 IFVLY 7

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**United States Patent****5,508,384****Murphy, et. al.****Apr. 16, 1996**

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**Polypeptide derived from a popamine receptor, and compositions and methods thereof****Inventors:** **Murphy; Randall B.** (Irvington, NY); **Schuster; David I.** (Wilton, CT).**Assignee:** **New York University** (New York, NY).**Appl. No.:** **118,270****Filed:** **Sept. 9, 1993**

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**Related U.S. Application Data**

Continuation-in-part of Ser No. 943,236, Sept. 10, 1992, abandoned.

**Intl. Cl.:****C07K 14/705****U.S. Cl.:****530/324****Field of Search:****514/12, 13, 2; 530/300, 324**

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**References Cited [Referenced By:]**

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**Other References**

FASEB, J. 3:1825-1832, May 1984, Strader et al. Structural Basis of .beta.-adrenergic receptor function.

Pharmac. Ther. 50:425-442, 1991, Jackson Structure and Function of G Protein Coupled Receptors.

Science 246:1310-1316, 03 Jun., 1988, Kobilka et al. Chimeric .alpha.(2),.beta.(2)-Adrenergic Receptors: Delineation of Domains Involved in Effects-Coupling and Ligand Binding.

Nature 336:783-787, 22 Dec. 1988, Bunzow et al, Cloning and Expression of a rat D(2) dopamine receptor cDNA.

*Primary Examiner:* Draper; Garnette D.

*Assistant Examiner:* Ulm; John A.  
*Attorney, Agent or Firm:* Browdy and Neimark

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### **Abstract**

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Compounds, compositions and methods involving purified, isolated and/or synthetic G-protein coupled receptor (GPR) polypeptides that comprise fragments, derivatives and/or consensus peptides of transmembrane domains of G-coupled receptor proteins, wherein the GPR polypeptide has biological activity selected from binding of a GPR ligand to a GPR or modulating the binding of a GPR ligand to a GPR.

**1 Claim, 22 Drawing Figures**

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[USPTO] [CNIDR]

CONTAINS UNPUBLISHED DATA  
Query= D6(14)B (NOT LDH) SP6 (SEE ALSO T7) TGGTTTAAAAATAATTAAACAAAAA  
(25 letters)

Database: Non-redundant GenBank+EMBL+DDBJ+PDB sequences  
272,299 sequences; 376,297,050 total letters.

Sequences producing High-scoring Segment Pairs:	High Score	Smallest Sum Probability		
		P(N)	N	
gb U32831 HIU32831	Haemophilus influenzae from bases 16...	101	0.64	1
emb Z50873 CEF17E5	Caenorhabditis elegans cosmid F17E5	101	0.64	1
gb U39742 CELC25F6	Caenorhabditis elegans cosmid C25F6.	101	0.64	1
gb M24056 DDITGVD	D.discoideum Val-tRNA gene, clone 1a...	98	0.81	1
emb X71399 LLNGS1G	L.luteus gene for glutamine synthetase	97	0.89	1
gb U41020 CELT27B1	Caenorhabditis elegans cosmid T27B1.	97	0.89	1
gb U44030 SCU44030	Saccharomyces cerevisiae chromosome ...	97	0.89	1
gb U39993 CELF47E1	Caenorhabditis elegans cosmid F47E1.	97	0.89	1
gb U39744 CELC03F11	Caenorhabditis elegans cosmid C03F11.	95	0.96	1
gb U40799 CELF42C5	Caenorhabditis elegans cosmid F42C5.	95	0.96	1
emb Z29443 CET07C4	Caenorhabditis elegans cosmid T07C4	93	0.992	1
emb Z73896 CEF09E8	Caenorhabditis elegans cosmid F09E8	93	0.992	1
gb U20682 MSU20682	Monoxia sp. mitochondrion 16S riboso...	92	0.994	1
gb M89984 HIVASPCR09	Human immunodeficiency virus type 1 ...	92	0.995	1
gb U36275 HVU36275	Hydra vulgaris cnidarian achaete-scu...	92	0.996	1
emb V00551 HSIFR9	Messenger RNA for human leukocyte (a...	92	0.996	1
gb I01602	Sequence 3 from Patent US 4810645	92	0.996	1
gb M23754 DDIUBIQG	D.discoideum ubiquitin mRNA, complet...	92	0.996	1
gb U51923 DDU51923	Dictyostelium discoideum STE20 (dkin...	92	0.996	1
gb L27469 DROP41C	Drosophila melanogaster (cDNA3) prot...	92	0.996	1
dbj D43635 DROEAGLE2	Fruitfly embryo mRNA for 'Eagle', co...	92	0.996	1
emb X07875 ECMCBEGF	E. coli genes mcbE, mcbF and mcbG fo...	92	0.997	1
dbj D43634 DROEAGLE	Fruitfly embryo mRNA for 'Eagle', co...	92	0.997	1
gb U38472 SPU38472	Schizosaccharomyces pombe essential ...	92	0.997	1
gb L31780 WALHPRT	Macropus robustus HPRT processed pse...	92	0.997	1
gb M19492 DDIUBIRPB	Slime mold (D.discoideum) ubiquitin ...	92	0.997	1
gb L36273 DRO25DC11Z	Drosophila melanogaster (subclone D2...	92	0.997	1
gb U07806 HSU07806	Human camptothecin resistant clone C...	92	0.997	1
gb I09478	Sequence 1 from Patent WO 8909222	92	0.997	1

gb|U32831|HIU32831 Haemophilus influenzae from bases 1631471 to 1645526  
(section 146 of 163) of the complete genome  
Length = 14,056

Plus Strand HSPs:

Score = 101 (27.9 bits), Expect = 1.0, P = 0.64  
Identities = 21/22 (95%), Positives = 21/22 (95%), Strand = Plus / Plus

Query: 4 TTTAAAAATAATTAAACAAAAA 25  
||||| |||||||||||||||  
Sbjct: 11216 TTTAGAAATAATTAAACAAAAA 11237

Query= D6(14)B (NOT LDH) SP6 (SEE ALSO T7) TGGTTTAAAAATAATTAAACAAAAA  
(25 letters)

Translating both strands of query sequence in all 6 reading frames

Database: Non-redundant GenBank CDS  
translations+PDB+SwissProt+SPupdate+PIR  
225,697 sequences; 63,534,404 total letters.

Sequences producing High-scoring Segment Pairs:	Reading Frame	High Score	Smallest Sum Probability	
			P(N)	N

Parameters:

V=100  
B=50  
H=0  
-filter=SEG  
P=4

# GLUCOSE TRANSPORT ACTIVITY QUIESCENT LACTATING

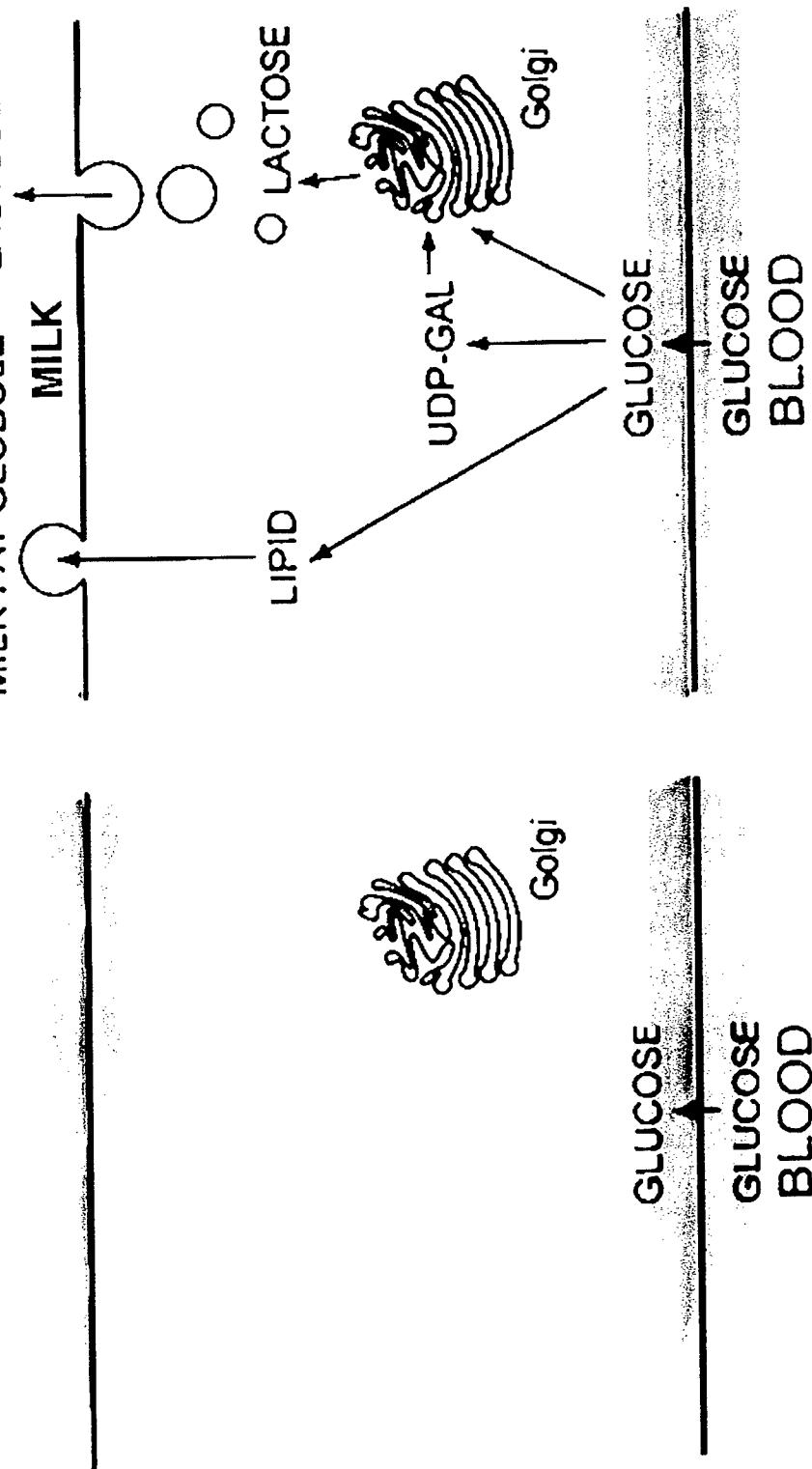


Figure 7



DEPARTMENT OF THE ARMY  
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND  
504 SCOTT STREET  
FORT DETRICK, MARYLAND 21702-5012

REPLY TO  
ATTENTION OF:

MCMR-RMI-S (70-1y)

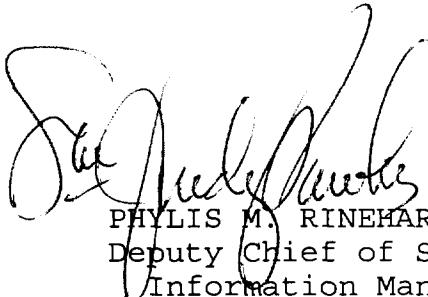
16 Jul 99

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2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by email at Judy.Pawlus@det.amedd.army.mil.

FOR THE COMMANDER:



PHYLIS M. RINEMART  
Deputy Chief of Staff for  
Information Management